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The role of a viral microRNA and RNA interference during viral replication in mammalian cells

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The role of a viral microRNA and RNA interference during viral replication in mammalian cells

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2012

Dedication

To Minhui Lim,

Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisor, Dr. Christopher Sullivan. I am so grateful for his continual support of my doctoral studies and research, for his patience, motivation, enthusiasm, and immense knowledge. Besides my advisor, I would like to thank to my thesis committee members: Drs. Edward Marcotte, Scott Stevens, Haley Tucker, and Marvin Whiteley for their encouragement and insightful comments. Their guidance helped me throughout all the research and writing of this thesis.

I am really fortunate to have been a member of Sullivan Lab and to work alongside such generous and brilliant people. My sincere thanks also goes to Sullivan Lab members: Chun Jung Chen, Jennifer Cox, Oliver Lin, Rodney Kincaid, James Burke, Lydia McClure, Thomas Penny, Rathi Kannan, Alysia East, Ryan Bass, Phanaksri Teva, Rathi Kannan, Houdah Abualtin, and Jennifer Harvey. I would especially like to thank Chun Jung Chen who collaborated to assay the luciferase activity to determine the MCV miRNA function detailed in Chapter 3. Thank you to Rodney Kincaid who was dedicated to his collaborative bioinformatics analysis for finding microRNA regulating interferon-stimulated genes. Thanks to Phanaskari Teva who contributed to the experiment of viral infection to observe miR-17 function in HSV-1 infectivity. And thank you to Jennifer Cox who performed microarray experiment to find interferon-stimulated genes under

miRNA regulation in Chapter 4. I am also thankful for the collaboration, in Chapter 2, received from Bethany O'Hara and Dr. Walter Atwood at Brown University.

I would like to express my heartfelt thanks to my family. Thank you to my parents, for bringing me into this world and supporting me spiritually throughout my life. Last but not least, I must express my great appreciation for a colleague, my wife: Minhui Lim. I could not finish this long way without her support and limitless love.

The role of a viral miRNA and RNAi during viral replication in mammalian cells

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The University of Texas at Austin, 2012

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RNA interference (RNAi) is an evolutionarily conserved process that regulates gene expression. Host cells and viruses interact in many ways, including through miRNAs and RNAi. Viral miRNAs are encoded when viruses, specially including the the polyoma and herpes families, are transcribed in the nucleus. Some viral miRNAs function to regulate host or viral gene expression. Most viral miRNAs' functions are not known, however, in great detail. A miRNA can be encoded late during infection, as it is by SV40, a model polyomavirus. This downregulates early viral gene expression by directing mRNA RISC-mediated cleavage. As more polyomaviruses are discovered that are associated with human disease, it becomes more important to understand their function and to uncover whether these emerging viruses encode miRNAs. The work presented here shows the discovery of several viral miRNAs in human polyomaviruses—JCV, BKV, and MCV. In addition, I found that viral miRNAs have the evolutionarily conserved function of negatively regulating viral early gene transcripts at a late stage in the infection. During viral replication, viruses utilize the miRNA components of RNAi. However, in invertebrate organisms RNAi also actively defends against viral infection. It is still being

debated, though, whether RNAi plays an antiviral role in mammalian cells. Should it be true that RNAi is an antiviral response in mammalian cells, then what is predicted by such a scenario is inconsistent with my studies. I have found that RNAi is strongly inhibited in the early stages after viral infection. Studies with a chemical mimic of viral infection (poly I:C) imply that the innate cellular immune response is responsible for this inhibition. I investigated the molecular changes, in response to viral infection, (e.g. poly ADP-ribosylation of Ago2) in the RNA-induced silencing complex (RISC). I determined that the inhibition of RNAi is brought about by components of the innate response. Completion of this study details a previously unknown "cross talk" between RNAi and the host innate immune response in mammalian cells. Furthermore, I found mir-17 family attenuates a subclass of interferon-stimulated genes. An understanding of viral miRNA and RNAi offers a clue as to we can use molecular intervention for viral infections.

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Chapter 1: Introduction

1.1 Viruses and RNA interference (RNAi)

The evolutionarily conserved process of post-transcriptional regulation of gene expression-directed by small RNAs (siRNAs or miRNAs), is known as RNA interference (RNAi) (Illustration 1) (Umbach and Cullen, 2009). Small RNAs enter a protein complex called the RNA-induced silencing complex (RISC) and bind directly to messenger RNA (mRNA) targets. This results in the cleavage of the transcript or specific inhibition of the translation of the transcript (Illustration 1B). It is believed that 70% of all human transcripts are regulated by miRNAs. Several DNA virus families— including the polyomaviridae and herpesviridae familes — also take advantage of this strategy for gene regulation and encode small RNAs called microRNAs (miRNAs) (Boss et al., 2009; Cullen BR 2009; Sullivan et al., 2005; Kim et al., 2009; Eash et al., 2006). The functions of the polyomaviridae and herpesviridae miRNA are still under investigation. However, several hypotheses exist for their function. One relevant to my work suggests that viruses encode miRNAs and utilize host RNAi machinery to evade the host immune response.

Whether RNAi plays an antiviral role in mammalian cells is a hotly debated topic (Umbach et al, 2009). Several reports show that mammalian viruses encode proteins that can inhibit RNAi activity (Bennasser et al., 2005, Haasnoot et al., 2007, Li et al, 2004). However, these studies were not conducted in the context of viral infection and involved only exogenous over-expression of the viral proteins. In addition, knocking out

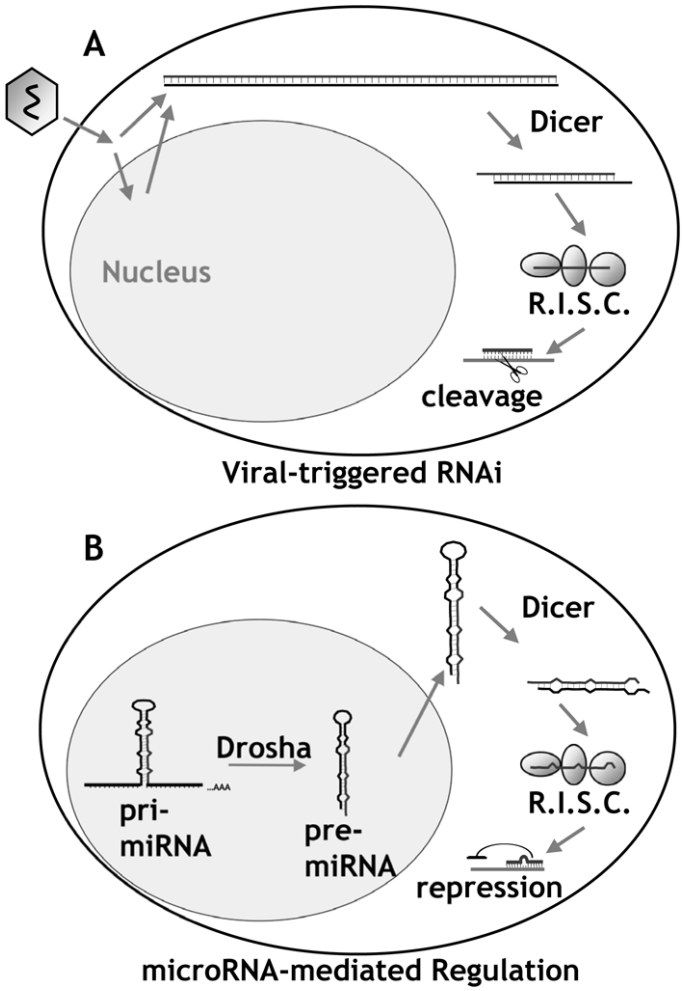

Illustration 1.1: RNA interference **A.** RNAi triggered by viral infection.
B. miRNA regulation of endogenous transcripts.

components of the RNAi machinery in mammalian cells increases the replication of some viruses. This is likely due to a loss of endogenous cellular miRNAs that are fortuitously complementary to viral transcripts (Otsuka et al., 2007). To date, no viral-specific siRNAs have been detected in studies conducted by small RNA cloning or though deep sequencing of viral infected mammalian cells (Umbach et al., 2010). At the same time, small siRNAs associated with the antiviral response are readily detectable in plants and insects during infection (Ding and Voinnet, 2007). Thus, greater understanding is needed concerning the role of RNAi as an antiviral defense in mammalian cells.

1.2 Polyomavirus-encoded miRNAs

Polyomaviruses are a family of small DNA tumor viruses. Both mouse and monkey polyomaviruses infections have been shown to cause tumorigenesis in the host. In the early 1970s, the human polyomaviruses (JCV and BKV) were discovered and have been implicated in several human diseases (Padgett BL, 1976; Knowles et al., 2003). Specifically, BKV induces nephritis in some kidney transplant recipients and JCV causes progressive multifocal leukoencephalopathy (PML) in immunocompromised patients suffering from AIDS. In 2008, a high throughput technique led to the discovery of a new polyomavirus (Merkel cell polyomavirus) that can cause an aggressive human skin cancer (Feng et al.,2008). MicroRNAs are regulatory small RNAs, consisting of 22 nucleotides. Researchers have focused on studying these small molecules; they are involved in the animal development, apoptosis, and human cancer. Five polyomavirus microRNAs have been identified. Recent work indicates that despite little sequence

similiarty, the polyomavirus miRNAs have an evolutionarily conserved function of regulating early viral protein production to help escape the host immune system (Grundhoff and Sullivan, 2011). As these miRNAs are functionally conserved, blocking miRNA function could be a useful strategy for therapeutic disease interventions in the future.

1.2.1 MicroRNA biogenesis

Small RNAs can serve as effectors to direct post-transcriptional regulation of gene expression (Cullen, 2009). Endogenous miRNAs arise from a series of nucleolytic processing events starting in the nucleus and into the cytosol by several RNase III endonuclease protein complexes. A class of small RNAs is called microRNAs and consists of approximately 21-23 nucleotides (Kim et al., 2009). In short, microRNAs are generated from hairpins transcribed by RNA polymerase II. The primary transcripts, called pri-miRNAs, are processed into 60-80 bp short hairpins known as pre-miRNAs by a microprocessor composed of Drosha/DGCR8 complex (Kim et al., 2009).

The Pre-miRNAs are exported into the cytosol from the nucleus by exportin-5. The short hairpins are then further matured into 22 nucleotide strands by the Dicer complex, composed of Dicer/TRBP (Chendrimada et al.,2005). The mature 22 nucleotides are finally incorporated into RNA–induced silencing (RISC) complex. The functions of miRNAs depend on the complementary sequences between the miRNA and 3'UTR of the target site. When there is a perfectly matched complementary sequence between the two strands, the target strand is cleaved in the middle of the binding site,

which is located 9-10 nucleotides from the 5' ends of the miRNA (Bartel, 2004). When there is an imperfect complementarity between the two strands, the target genes are typically translationally repressed by the RISC complex. Researchers have recently identified over 1000 human miRNAs. These miRNAs are involved various processes, including the immune response, apoptosis, animal development, and cancer (Kim et al., 2008).

In the field of virology, diverse viruses, including an ascovirus, several members of the Herpesviridae and Polyomaviridae and Retrovirus, have been shown to encode miRNAs that autoregulate viral gene expression (Hussain, Taft, and Asgari, 2008; Gottwein and Cullen, 2008; Pfeffer, 2008, Rodney e al., 2012). Reports that HIV also encodes siRNA are being debated. The following herpesvirus family encodes miRNAs: human cytomegalovirus (CMV), Herpes Simplex Virus (HSV-1, 2), Kaposi's sarcoma-associated herpesviruses (KSHV), and Epstein Barr virus (EBV) (Cullen, 2006). Polyomaviruses with varying host ranges also encode miRNAs: from murine (MuPV) andmonkey (Simian Vacuolating virus 40) to human polyomaviruses (BKV, JCV and MCV) (Sullivan et al., 2008; Seo et al., 2008; Seo et al., 2009).

1.2.2 The Polyomavirus family

Polyomaviruses have been used to discover important tenants of molecular biology such as alternative splicing and mechanisms of oncogenesis. Polyomaviruses consist of a family of small, naked capsid, circular, double-stranded DNA genomes (Sullivan et al., 2005). The polyomavirus family has a narrow host range in which each

virus can replicate actively in the susceptible species. The polyomavirus family also has a highly conserved genome structure for gene expression. The approximately ~ 5 kilobase, closed genome has an early transcript expressed in one direction and the late transcript expressed in the other direction. T antigen translated from the early transcript is a well-studied oncoprotein involved in tumorigenesis.

The mouse polyomavirus has been a good *in vivo* model for the study of tumorigenesis. It expresses middle T antigen— distinct from the other polyomavirus families. The SV40 virus, discovered 50 years ago, has been a standard model virus of polyomaviral biology. As an old-world primate polyomavirus, it was identified as a contaminant of the polio vaccine. Although mostly discredited, some scientists continue to debate whether the polio vaccine leads to the occurrence of cancer in the human population. Nevertheless, SV40 is still broadly used for studying polyomavirus biology as an etiological agent for cancer.

In 1971, two human polyomaviruses, JC virus and BK virus, were discovered (Eash et al., 2006). Both are related to serious human diseases in immunocompromised patients. Over 70% of the human population tests positive in serological testing Researchers recently identified three human novel polyomaviruses (Eash et al., 2006). Both Karolinska Institutue (KI) and Washington University (WU) virus were isolated from respiratory tract samples. It remains to be seen, however, whether these viruses are closely related as etiological agents for respiratory disease.

In 2008, the first polyomavirus associated with human cancer was identified with new, molecularly-based high throughput technology (Feng et al., 2008). The follow-up

papers demonstrated a number of findings. First, the virus is closely linked with the aggressive skin cancer, Merkel cell carcinoma; 80% of clinical samples contain an intergraded copy of the Merkel Cell Polyomavirus genome. In addition, permanent viral integration into the genome is essential for skin cancer development.

1.2.3 Polyomaviral-encoded miRNAs

Over the last eight years, miRNAs have been isolated from different species-specific polyomaviruses. Using bioinformatics approaches combined with northern blot analysis, two polyomaviral miRNAs were detected from each species of polyomavirus—murine polyomavirus, SV40, three human polyomaviruses (BKV, JCV and MCV)(Sullivan et al., 2005; Seo et al., 2008; Seo et al., 2009). Interestingly, both miRNAs are processed from the same precursor and have distinct functions from cellular miRNAs. As part of the polyomavirus life cycle, these viruses use the host machinery to generate miRNAs as a strategy for the regulation of gene expression.

The primary transcripts synthesized from the host polymerase II are processed into ~80 nucleotide host microRNA processors even though each virus has a minutely different miRNA in length. miRNA deep sequencing has mapped the 5′ and 3′ ends of miRNA, indicating that these viruses have a majority of 22 nucleotide-matured miRNAs with a minor proportion with 21 nucleotides. These short variants are most likely generated from alternative two steps of miRNA biogenesis and it is questionable whether these viral miRNA variants will have any distinct function.

Interestingly, although possessing little or no sequence identity, polyomaviral

miRNA genomic locations are positionally conserved. The mouse and MCV polyomaviral miRNAs are located near to one another in the genome alternatively to the SV40-related miRNAs. Considering that the miRNA sequences are not very similar, positional conservation is intriguing. The MCV miRNAs are isolated from two different isotypes of Merkel cell polyomaviruses (Seo et al, 2009) whose precursors have a polymorphism in the hairpin but not in the miRNA sequences. The strictness in sequence composition of the hairpin sequences strongly affects the efficiency/biogenesis of miRNAs, as evidenced by experimental mutation (Gottwein et al., 2006). However, the polymorphic change in MCV pre-miRNA affects neither the biogenesis of the miRNAs nor the function of targeting mRNAs. Even though the sequences among several polyomaviral miRNAs are different (group I: SV40, JCV, BKV miRNAs, group II: muPy, MCV miRNAs), the miRNAs' location is highly conserved. This implies that viral miRNAs are not only important to the virus, but there is evolutionary pressure to be functionally conserved across viral families.

1.2.4 Polyomaviral miRNA function

The polyomaviral late transcript is encoded antisense to the early transcript in the viral genome (Sullivan et al., 2005). The miRNAs are expressed from the late transcript and, therefore have perfect complementarity to their own target transcripts. Polyomaviral miRNAs are enriched in the late viral lytic infectious cycle, even though the expression mechanism remains to be determined. Current hypotheses include miRNA generation by a polyadenylation read-through mechanism during the late infection. More specifically,

miRNAs are more often processed from long, non-coding read-through transcripts of late transcripts (Chen et al., personal communication). Thus, polyomaviral miRNAs are enriched late during infection. Due to their perfect complementarity to the target transcript, polyomaviral miRNAs act in a siRNA-like function to cleave the target transcripts in the middle of the miRNA binding site. The cleavage sites can be determined using two separate methods: modified 5' RACE and RNAase protection assay. Cellular miRNAs, in only rare cases cleave their target transcripts, but instead inhibit translation. The polyomaviral miRNAs cleavage mechanism, similar to siRNA-like regulation, is comparable to herpesviral miRNAs (Sullivan et al., 2005; Seo et al., 2008). Many questions remain in the study of polyomaviral-encoded miRNAs including, what is the role of miRNAs specifically expressed at certain times during viral replication Mutant viruses that lack viral miRNA expression have an intact infectious cycle and replication cycle in *in vitro* cell culture conditions. However, Sullivan et al. (2005) have shown reduced Large T antigen protein expression and a decrease in viral cytotoxicity during infection. This suggests that the miRNAs have evolved to tightly tune the level of its own protein possibly to evade the host immune system. During replication, large T antigen is multifunctional and also is exposed to the host adaptive immune system. Knocking down large T antigen expression late during infection via miRNA regulation might act as an escape mechanism from host-immune surveillance. All of the polyomaviral miRNAs currently isolated show autoregulation of early transcripts, indicating that such a strategy might be necessary for the polyomavirus life cycle. If the polyomavirus acts with a siRNA-like function for autoregulation, is there any interaction between polyomaviral

miRNAs and cellular messenger RNAs? It remains to be seen whether these miRNAs target cellular messenger RNAs. One possible scenario is that it also functions as a miRNA to regulate the host messenger RNAs. However, even with their conserved function, polyomaviral miRNA sequences differ, including changes in the seed region, which is an important module for translational control. How do these viral miRNAs, with different sequences, possess a conserved function to regulate the same target genes? One possibility is that these polyomaviral miRNAs might bind to a different 3' UTR region sharing the same target. It is questionable, however, that a functional strictness makes it possible to evolutionally change in the sequence of miRNAs in the virus. Alternatively, the different polyomaviral miRNAs could have a different target repertoire likely due to a strain specificity showing cellular tropism. For example, a recent paper showed JCV and BKV miRNAs commonly regulate the stress response gene to mute the host's immune response gene (Bauman et al., 2011). New techniques to search for the viral miRNAs cellular targets, such as HITS or PAR-CLIP, have been well developed and used increasingly to identify novel functions for miRNAs. Any future cellular targets identified for the different polyomaviral miRNAs will be helpful in providing a better understanding of the miRNAs'function (Ziegelbauer et al., 2009).

1.3 RNAi as an antiviral mechanism in plant and invertebrate animals

RNA silencing as an antiviral defense is widely accepted in both plants and invertebrate animals (reviewed in Ding and Voinnet, 2007). DNA viruses make long,

double-stranded RNAs through overlapping transcription. RNA viruses also produce double-stranded RNAs during infection. Virus-derived small interfering RNAs (viRNAs) are produced during infection and leads to the induction of specific gene silencing by viRNA-RNAi effector complexes. The key component of antiviral RNA silencing is well characterized in plant, flies, and worms using biochemical and genetic approaches. Furthermore, viral suppressors for gene silencing are expressed as a countermeasure to the host's defenses. In plants, some viruses induce severe viral disease symptoms (ring spots) early during viral infection but then gradually disappear (Reviewed by Baulcombe). Newly emerging leaves are immune to this same virus infection, implying a protection of viral spread. This phenomenon was referred to as a viral infection "recovery" before it was established that this is an example of antiviral immunity directed by RNA silencing.

In the model organism, Arabidoposis thaliana, the antiviral defense of RNAi, mostly relies on Dicer-like proteins (DCL2-4) (Bartel, 2004). It is known that DCL2-4 produces small interfering RNAs (siRNAs) with lengths from 21-24 nucleotides (Brodersen and Voinnet, 2006). These are made from a virus derived from long double-stranded RNAs (dsRNAs). The respective mutations in the four Arabidopsis DCLs do not show critical changes in viral susceptibility because of the functional redundancy of the DCL proteins. Ago1 is a major antiviral slicer, but other Ago proteins are likely to have a function controlling the viral gene's translation. More than 35 individual VSR families reveal that plant viruses ubiquitously possess a counter-measure for host antiviral RNA

silencing (Li and Ding, 2006). For example, a Cymbidium ringspot virus suppresses gene silencing by sequestering viRNAs and prevents their incorporation into RISC (Lakatos et al., 2004).

In addition, the *C elegans* Dicer complex processes siRNAs and miRNAs to play an important role in defending adult worms from VSV, which naturally infects livestock. Lastly, Felix et al., (2006) identified the first virus, a nodavirus (Orsay virus), to naturally infect worms. Of significance, Orsay virus easily infects *C. elegans* that are defective in RNAi. This virus expressed higher levels of viral RNA when compared to infection of the corresponding wild-type worms.

In Drosophilia, long double-stranded RNAs produced by viruses are recognized by the RNase III enzyme called Dicer-2, and are effectively converted into 21 nucleotide siRNAs (Hammond, 2005). The proof of RNAi as a natural antiviral defense in Drosophila has been shown using several genetic studies. The Dcr-2, R2D2 mutant flies are highly susceptible to fruit viruses compared to wild type flies. Van Rjj et al, (2006) show that Ago-2 is critical for antiviral defense in adult flies. Ago-2 defective flies are susceptible to infection with several fly viruses including, DCV and CrPV. Antiviral RNAi in flies is counteracted by suppressors encoded by virus. DCV virus expresses a suppressor which prevents the conversion of long double-stranded RNAs into 21 nucleotide siRNAs.

1.4 Mammalian antiviral mechanism

Mammalian cells recognize viral invasion and activate their sophisticated self-defense mechanism called the adaptive immune response. This results in limiting viral replication and subsequent secondary infections. The recognition of pathogen invasion is initiated by pattern recognition receptors (PRRs) (Akira et al., 2006; Beutler et al., 2007; Medzhitov et al., 2007). PRRs sense pathogens associated with molecular patterns: DNA, single or double-stranded RNA structure, RNA with 5'-triphospate ends, and viral proteins. Researchers have experimentally explored several PRRs: membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors, and cytosolic receptors, named RIG-I like receptors (RLRs), and Nod-like receptors (NLRs). TLRs and RLRs play crucial roles in the induction of interferon or the inflammatory cytokine response to viral infections (Petrilli et al., 2007; Kanneganti et al., 2007). Cytosolic RIG-I receptors typically recognize a viral intermediate during viral replication such as viral RNA with 5' triphosphate or double-stranded RNAs. Such recognition mediates signals through MAVS/IPS-1 and transduces the signal through NF-kappa B or IRF-3. This leads to type I interferons and inflammatory cytokines that activate the innate or adaptive immune responses. Interferons increase genes involved in the dsRNA signaling pathway. Double-stranded RNAs are recognized by PKR (Garcia et al., 2006). Activated PKR phosphorylates eIF2 alpha, causing global translational inhibition. Another signaling protein, OAS, recognizes double-stranded RNAs and synthesizes 2'-5' oligoadenylates. RNAase L, bound to this molecule, is activated and restricts viral replication through viral or cellular RNA cleavage (Clemens et al., 2005). Lastly, RNAase L also activate a

MAVS-signaling pathway to increase endogenous double-stranded RNAs, however, this process is not well characterized (Malathi et al., 2007).

Type I interferons, such as interferon alpha and interferon beta (IFN-alpha and beta), convey signal transduction through a Janus Kinase (Jak) and are an activator oftranscription (STAT) pathway to stimulate gene expression (Illustration 1.2) (reviewed by Murray, 2007). After stimulation with type I interferons, the receptors dimerize. Jak1 and Tyk2 associate with interferon receptors to phosphorylate STAT1 on tyrosine 701 and STAT2 on tyrosine 690 (Banninger and Reich, 2004). Phosphorylated STAT1 and STAT2 translocate into the nucleus and bind to interferon regulatory factor 9 (IRF9) to canonically form IFN stimulated gene factor 3 (ISGF3) (Tsuno et al, 2009). ISGF3 binds to the IFN-stimulated response element (ISRE) present on the promoters of interferon responsive genes and leads to enhanced transcription. An interferon signal transduction pathway increases the expression of several hundred genes and postulates the antiviral state. Several interferon-stimulated genes encode enzymes that have been extensively studied. These include PKR, OAS (described above), and Mx. Mx genes express large GTPases related to dynamin and limit the broad range of RNA virus replication recognizing nucleocapsid-like proteins (Martens and Howard, 2006; Haller et al., 2007). Interferon-stimulated factors lead to changes in cellular translation, cell cycle arrest, and apoptosis. The type I interferons also activate the innate immune-cell specific responses to promote maturation of Dendritic cells and up-regulation of NK cells activities (Nguyen

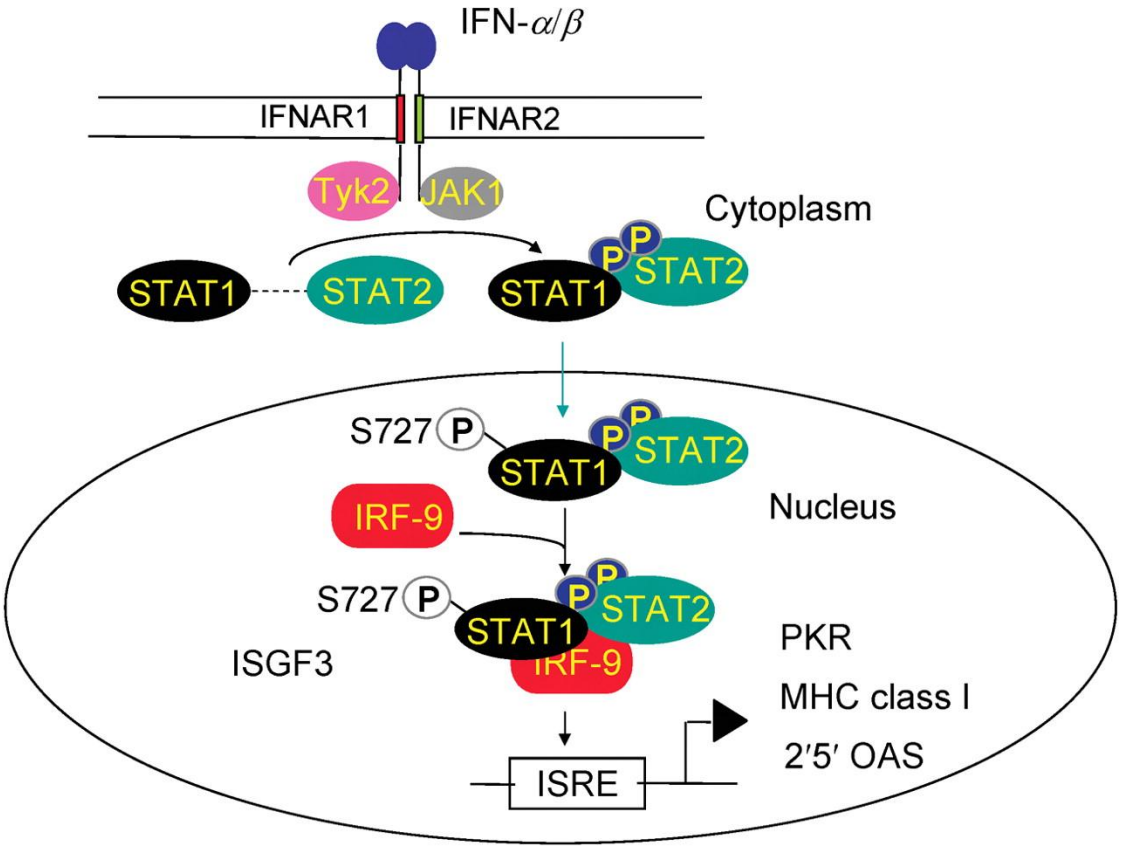

Illustration 1.2 Signalling pathway activated by IFN-*α*/*β*. The biological activities of IFN-*α*/*β* are initiated by binding to the type I IFN receptor. This leads to the activation of the receptor-associated tyrosine kinases JAK1 and Tyk2, which phosphorylate STAT1 on tyrosine 701 and STAT2 on tyrosine 690. Phosphorylated STAT1 and STAT2 interact strongly with each other by recognizing SH2 domains, and the stable STAT1–STAT2 heterodimer is translocated into the nucleus, where it interacts with the DNA-binding protein IRF-9. The IRF-9–STAT1–STAT2 heterotrimer is called ISGF3 and it binds to a sequence motif (the IFN-stimulated response element or ISRE) in target promoters and brings about transcriptional activation. In addition to the phosphorylation of tyrosine, STAT1 also requires phosphorylation on serine 727 for function.

et al., 2002). One important function of interferon is to induce pro-apoptotic genes in interferon-treated cells which dramatically occur after detection of viral PAMP signatures such as double-stranded RNAs. Several pathways have been suggested for how interferon is involved in the induction of apoptosis (reviewed by Clemens, 2003; Maher et al., 2007) 1) interferon induces pro-caspase genes, and 2) PKR, OAS, and p53 induced by interferon can give rise to apoptosis after double-stranded RNA treatment or viral infection. Nevertheless, the molecular mechanisms have yet to be fully defined and remain undetermined.

1.5 The role of RNAi in the antiviral defense of mammalian cells

In mammalian cells, transfection with small interfering RNAs (siRNAs) complementary to the viral genome efficiently limits viral replication and protects the host from lytic viral infection. Initial hypotheses were that RNAi may function as an antiviral mechanism in mammalian cells. However, antiviral defense by artificial siRNAs cannot prove that RNAi is a naturally occurring, intrinsic antiviral response. A major question remains whether mammalian cells are able to induce RNAi in response to long double stranded RNAs, a signature viral intermediate. In early pioneering work on RNAi, RNA silencing was shown to be effective in germ cells such as oocyte, or nondifferentiated mouse cells, and mouse embryonic stem cells grown in cell culture (Babiarz et al., 2008; Tam et al., 2008). However, RNAi elicitation from long double stranded RNAs is not effective in somatic cells. The difference in capability of RNAi elicitation between germ and somatic cells is also recently confirmed by *in vivo*

transgenic mouse models, using exogenously expressed regulatory RNA with a long hairpin structure (Nejepinska et al., 2012). The long, double stranded hairpins are robustly processed into small RNAs in germ cells and reduce the expression of cognate messenger RNA sequences, while RNA silencing from the same reporter genes were not down regulated in somatic cells. There are several hypotheses for why RNAi has differing responses to long double stranded RNAs in somatic cells. Long double-stranded RNAs induce nonspecific effects, such as PKR and RNase L affect global control of gene expression and changes in cellular RNA levels. It is noteworthy that the interferon pathways are deficient in ES cells and germ cells.

The second question is if siRNAs of viral origin are generated from virus-infected mammalian cells. Pfeffer et al., (2005) attempted to identify viral small RNAs from cell lines infected with a broad range of viruses, DNA viruses, including KSHV, MHV68, HCMV, as well as RNA viruses including YFV and HCV. From cell lines infected with DNA viruses, they found newly discovered miRNAs but failed to detect any siRNAs. Researchers including Sullivan group have recently conducted comprehensive research for viral small RNA by taking advantage of next-generation sequencing technologies (Lin and Sullivan, 2011). Recently, the Cullen group reported that small RNAs from DNA or RNA viruses are not comparable to functional siRNAs shown by plants and invertebrates (Umbach et al., 2010). Other groups have reported that some viral-derived small RNAs from six RNA viruses (Hepatitis C, Polio, Dengue, Vesicular Stomatotis, West Nile viruses) may function as a component that interacts between viruses and host

(Parameswaran et al., 2010). However, it is still unclear that the generated viral small RNAs are RNAi effectors, which are dependent on the Dicer enzyme for the process. As small RNA production is partially reduced in the Dicer-depleted cells, some viral small RNAs can be derived from random degradation or digestion by unknown RNase. Therefore, it still remains to be determined whether the small RNAs derived from viruses in mammalian cells have a capacity to induce antiviral RNAi, as siRNAs derived from viruses in plants and invertebrates.

The third question is whether mammalian viruses express RNA-silencing suppressors. Some reports show that mammalian viral proteins can inhibit RNAi activity. Influenza NS1, vaccine virus E3L, Ebola virus VP35, HIV-Tat, and primate foamy virus (PFV) Tas have been suggested to work as mammalian suppressors of gene silencing (Li et al., 2004; Haasnoot et al., 2007; Bennasser et al., 2005; Lecellier et al., 2005). These studies, however, were mostly not conducted in the context of infection and only involved exogenous over-expression of proteins. Because it has been demonstrated that even prokaryotic dsRNA binding proteins can nonspecifically inhibit mammalian RNAi in the condition of overexpression (Lichner et al., 2003), it is uncertain whether these proteins act as RSSs during *bona fide* infection. There has been one experiment in the context of HSV-1 virus infection (Wu et al., 2009). This virus inhibited RNA interference and the authors hypothesized that the viral protein may inhibit RNA interference. However, they failed to prove that RNAi is blocked by a protein such as a viral suppressor.

The last question is whether endogenous cellular miRNAs can prevent viral replication. It has been suggested that mammalian cells mount cellular miRNAs to inhibit viral replication where host miRNAs are imperfectly matched with viral transcripts. Evidence as part of this argument is that mammalian miRNAs are evolutionary conserved and sequence change is constrained because changes in the seed sequence might transform the milieu of each targetome. Whereas, when the RNA virus mutation rate is considered to be fast, the cellular defense using germ line transmitted miRNAs is weak in the arms race between virus and host, from the perspective of viral evolution. Nevertheless, it has been described that a Dicer hypomorphic mouse is hyper susceptible to VSV infection because of the loss of its endogenous cellular miRNA, which is complementary to viral transcripts (Otsuka et al., 2007). A less amount of Dicer reduces cellular mir-24 and mir-93, controlling VSV M gene expression. Even though this result proposes the idea that cellular miRNAs can act as an antiviral defense, it has the weakness that the virus is not a naturally infectious pathogen for mice. A recent paper reported that the experiment infected with physiologically relevant MCMV herpes virus was less infected at an early phase of infection using the same Dicer hypomorph mouse (Ostermann et al., 2012). The author claimed that some interferon-stimulated genes are upregulated in Dicer hypomorph cells. This suggests that the host's antiviral response, the interferon-stimulated gene, is regulated by miRNAs. Therefore, in spite of diverse research into the antiviral mechanism of RNAi in mammalian cells, gaps have been exposed in the original hypothesis. Unbiased, continued research will further develop

how viruses interact with host RNAi and open new questions regarding the role of RNAi during viral infection and host response in mammalian cells

1.6 Antiviral defense and the regulation of miRNA activity by cytosolic PARPs.

Recent reports indicate an important role is played of macromolecule poly(ADP-ribose) pADPr in DNA damage repair, chromatin remodeling, and transcription in the nucleus of eukaryotic cells (Schreiber et al., 2006). The pADPr forms a scaffold to recruit base excision repair (BER) factors to the DNA damage site and facilitates DNA repair by relaxing the chromosome structure. However, Leung et al., recently discovered pADPr has a post- transcriptional regulatory function in cytoplasm (Illustration 1.3). Under stress, cytoplasmic PARP complex aggregates around each other by poly ADP-ribosylation modification. The PARP enzymes also modify miRNA-binding proteins (Ago1-4), RNA decay factor G3BP1, and translational suppressor TIA-1. Interestingly, cytoplasmic proteins in stress granules are poly ADP ribosylated under cellular stress. The modification of Argonaute protein results in a decrease in global miRNA activity. Immunofluorescence and co-immunoprecipitation experiments show PARP5, PARP12, and PARP-15 in stress granules are co-localized with Ago2 protein. Even though the biological function of cytosolic PARPs has yet to be investigated, PARP12 is an interferon-stimulated gene (ISG) and exhibits ADP-ribosyltransferase activity. Recently it has been shown that the long isoform of PARP12 displays antiviral activity for a broad range of alphaviruses and RNA viruses (Atasheva et al., 2012). PARP 13 was originally

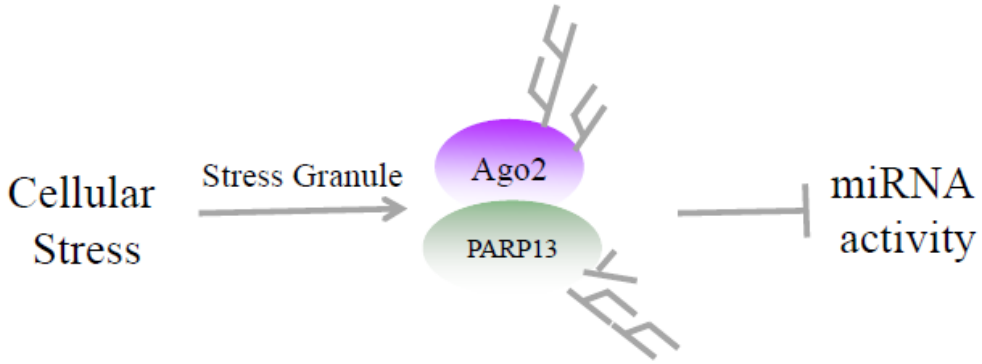

Illustration 1.3 Poly(ADP-ribose) regulates stress responses and miRNA activity in the cytoplasm. Poly(ADP-ribose) is a major regulatory macromolecule in the nucleus, where it regulates transcription, chromosome structure, and DNA damage repair. Functions in the interphase cytoplasm are less understood. poly(ADP-ribose) in the assembly of cytoplasmic stress granules, which accumulate RNA-binding proteins that regulate the translation and stability of mRNAs upon stress. poly(ADP-ribose), six specific poly(ADP-ribose) polymerases, and two poly(ADP-ribose) glycohydrolase isoforms are stress granule components. A subset of stress granule proteins, including microRNA-binding Argonaute family members Ago1-4, are modified by poly(ADP-ribose), and such modification increases upon stress, a condition when both miRNA-mediated translational repression and microRNA-directed mRNA cleavage are relieved. (Illustration from Leung et al., 2011)

characterized as ZAP, a host factor restricting the retrovirus infection (Gao et al., 2002). The antiviral activity of PARP 13 does not arise through auto-polyADP-ribosylation; it has a lack of PARP domain or criticial residue for the ADP-ribosylating activity (Hottiger et al., 2008). Researchers have speculated that PARP13, defective of PARP activity, can be poly ADP-ribosylated by other cytosolic PARP enzymes, resulting in the inhibition of miRNA activity. It has recently been shown that PARP13.2, the shorter isoform between two isoforms of PARP13, binds to RIG-I in response to viral infection and stimulates type I interferon and inflammatory cytokines (Hayakawa et al., 2011). It also shows that the long form of PARP13 (PARP13.1) has stronger antiviral activity in a PARP-like domain (Kerns et al., 2008).

1.7 Dissertation Objective

RNA interference (RNAi) is an evolutionarily conserved process that regulates gene expression. Host cells and viruses interact in many ways, including via miRNAs and RNAi. Viruses from both the polyoma and herpes families are transcribed in the nucleus and encode viral miRNAs. Several studies have shown that some viral miRNAs function to regulate host or viral gene expression, but what remain unknown are the molecular functions for most viral miRNAs. SV40, a model polyomavirus, encodes a miRNA late during infection that downregulates early viral gene expression by directing mRNA RISC-mediated cleavage. As more polyomaviruses are discovered that are associated with human disease, it is important to understand these emerging viruses' functions and to uncover whether they encode miRNAs. Although the miRNA components of RNAi

are utilized by viruses during viral replication, RNAi also serves to actively defend against viral infection in invertebrate organisms. However, it is debated whether RNAi plays an antiviral role in mammalian cells. What is needed here is clarity regarding the role of RNAi as an antiviral defense in mammalian cells.

In Chapters 2 and 3, I describe how human polyomaviruses encode miRNAs and explaine that they utilize them to auto-regulate viral antigens. Small RNAs have already been implicated in numerous and diverse processes including neuronal function, diabetes, developmental timing, and cancer. Previous studies have shown that several diverse families of viruses with DNA genomes encode miRNAs. However, the functions of the vast majority of viral-encoded miRNAs are unknown. I discovered that several different members of the Polyomavirus family encode miRNAs, all with a common function. The Polyoma family is responsible for several serious diseases found mostly in immunocompromised or elderly patients. These diseases include kidney disease, loss of cognitive function, and a particularly malignant type of skin cancer called Merkel cell carcinoma. I applied a computational miRNA discovery approach to screen various members of the Polyomavirus family for candidate miRNAs. From this list of candidates, I utilized molecular biological techniques to validate *bona fide* miRNAs encoded by JCV (etiologic agent of neural disease), BKV (etiologic agent of kidney disease), and MCV (associated agent of tumorigenesis). The BKV and JCV miRNAs share a common genomic location and display a high degree of sequence similarity. Interestingly, MCV encodes a miRNA in a different genomic region that shares no sequence identity with

JCV or BKV. Despite these differences, it shows that the miRNAs from all three viruses share a common function to down-regulate early viral gene expression during late phases of infection. A previous study showed that this type of autoregulation in SV40, a related common laboratory model of Polyomavirus, can contribute to immune evasion. Therefore, blocking the function of the identified human Polyomaviral miRNAs may be a valid strategy for future therapeutic interventions.

In Chapter 4, I describe how in mammalian cells RNA interference plays a role during viral replication. Contrary to what would be predicted if RNAi were an antiviral response in mammalian cells, my studies show that RNAi is strongly inhibited at early time points after viral infection. Studies with a chemical mimic of viral infection (poly I:C) suggest that the innate cellular immune response is responsible for this inhibition. I described the molecular changes (e.g. polyADP-ribosylation of Ago2) in the RNA-induced silencing complex (RISC) in response to viral infection. I determined which components of the innate response affect the inhibition of RNAi. Furthermore, I proposed that inactivation of RNA interference promotes the expression of interferon-stimulated genes. Specifically, miR-17 increases the infectivity of HSV-1, muting the interferon response genes induced by type I interferon. I experimentally show that an ISG can be regulated by miR-17/93 and one ISG, IRF9, is derepressed by a trigger from the antiviral response.

Chapter 2: Evolutionarily conserved function of a viral miRNA

2.1 INTRODUCTION

MicroRNAs (miRNAs) are small, ~22-nucleotide RNA molecules that regulate gene expression (Bartel, 2004). miRNAs bind to an mRNA and can repress translation or direct the cleavage of the target mRNA as part of the multiprotein RNA-induced silencing complex (RISC). The so-called seed region (nucleotides 2 to 8 of the 5′ ends of miRNAs) plays an important role in target selection by RISC-bound miRNAs (Fillipowicz et al., 2008). Host-encoded miRNAs have been shown previously to play a role in processes relevant to viral infection, such as apoptosis and the adaptive and innate immune responses (Lodish et al., 2008). Additionally, members of several virus families have been reported to encode miRNAs (Cullen, 2006; Grey et al., 2008; Peffer et al., 2003; Samols et al.,2006; Sarnow et al.,2006; Sullivan and Ganem, 2005). Activities have been ascribed to a few such miRNAs; however, the functions of the majority of virus-encoded miRNAs remain poorly understood.

We have shown previously that the monkey polyomavirus simian virus 40 (SV40) encodes a pre-miRNA late during infection that is processed into two miRNAs (Sullivan et al., 2005). Both SV40-encoded miRNAs function to downregulate the expression of the viral early genes by directing their RISC-mediated cleavage. The two human polyomaviruses JC virus (JCV) and BK virus (BKV) cause significant morbidity and mortality in immunosuppressed patients (Berger, 2007). JCV is the causative agent of a

fatal central nervous system demyelinating disease, progressive multifocal leukoencephalopathy (PML). BKV is the causative agent of polyomavirus-associated nephropathy in renal transplant patients. Currently, there are no drugs that are effective against polyomaviral infection. In this report, we now show that the human polyomaviruses BKV and JCV also encode miRNAs. Interestingly, results from fine-mapping studies show that the JCV and BKV miRNAs contain multiple differences in their seed sequences compared to the seed sequences of the SV40 miRNAs. Despite this finding, these miRNAs show similarities to the SV40 miRNAs in processing and share a conserved autoregulatory function with the SV40 miRNAs. The evolutionary implications of this finding are discussed below.

2.2 RESULTS

2.2.1 JCV and BKV encode miRNAs homologous to SV40 miRNAs.

Previously, using the viral miRNA prediction software vMir, we predicted that JCV and BKV would encode pre-miRNAs homologous to SV40 pre-miRNA (Sullivan et al., 2005). However, given the high false-positive rate of vMir and the fact that the predicted candidates were only ~65% identical, it was unclear if these predictions were valid (Grunhoff et al., 2006). We utilized a newer version of vMir (Grundhoff et al., 2006; Sullivan and Grundhoff, 2007) to examine the JCV and BKV genomes for candidate pre-miRNAs. This analysis identified four and two candidates for the BKV and JCV genomes, respectively (data not shown). Next, we looked for the possible derivatives

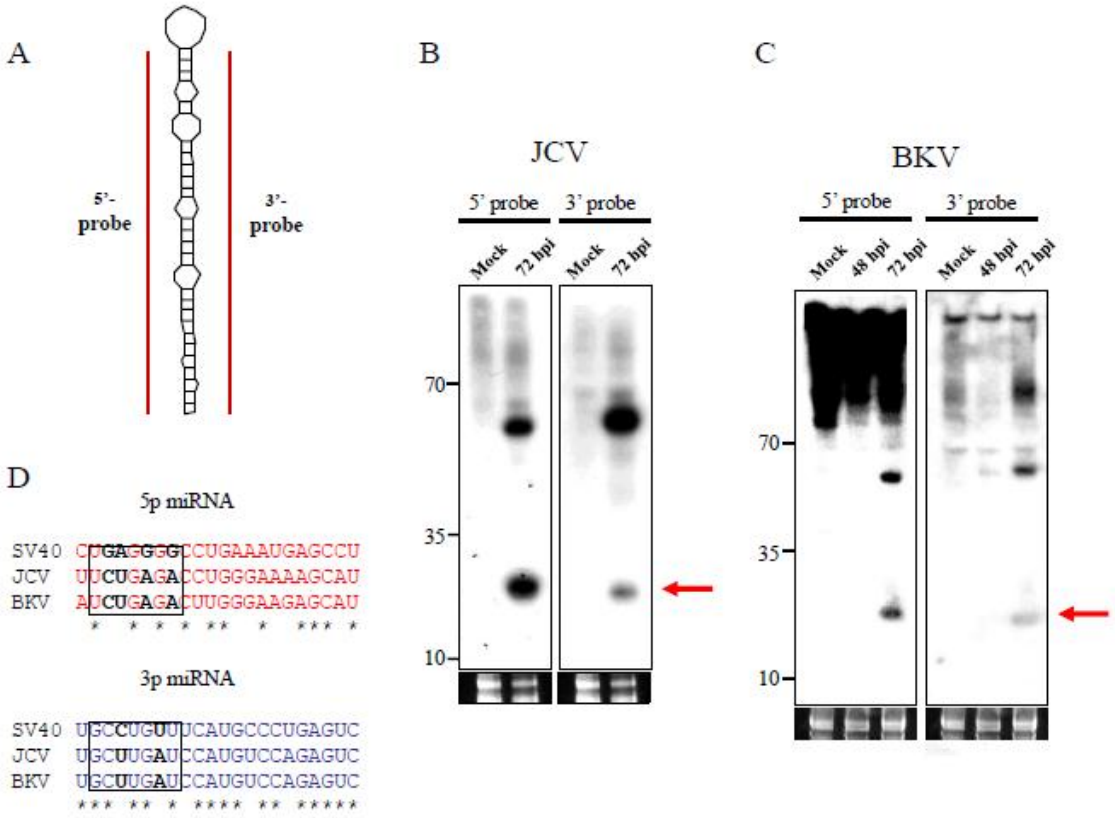

Figure 2.1 JCV and BKV encode miRNAs homologous to the SV40 miRNAs. (A to C) Northern blot analyses of the JCV and BKV miRNAs. (A) Diagram of probes used. (B) JCV miRNAs. (C) BKV miRNAs. The arrows indicate the bands corresponding to the miRNAs. Ethidium bromide staining of the low-molecular-weight RNA is shown as a loading control. Mock, uninfected. hpi, hours postinfection. (D) Alignment of the BKV and JCV 5p and 3p miRNAs with those of SV40. The 5p and 3p miRNAs are aligned with asterisks marking the conserved nucleotides. Note that the seed region (boxed; nucleotides 2 to 8), which is essential for target recognition, is not conserved. Nucleotides that differ among the SEED sequences are in bold.

from each predicted pre-miRNA via Northern blot analysis. Individual 5p and 3p probes were generated for each candidate, and Northern blot analysis was conducted (Figure 2. 1A). Only those candidates that showed sequence identity to SV40 miRNAs scored positive, and similar to SV40 miRNAs, these candidates were easily detected only at late times postinfection (Figure 2.1B and C; also data not shown). Notably, for all three viruses, both arms of the pre-miRNA are processed into detectable miRNAs. Interestingly, like SV40,

JCV and BKV each have a readily detectable band corresponding to the pre-miRNA. This results in a relatively higher ratio of the ~60-nucleotide pre-miRNA band to the processed ~22-nucleotide miRNA band than is detected for most cellular miRNAs (Fig. 2.1B and C) (Sullivan et al., 2005). Taken together, these results suggest that both JCV and BKV encode pre-miRNAs with notable similarities to SV40 pre-miRNA.

To map the derivative miRNAs with more precision, we generated a small RNA library from cells infected with either virus. To do this, we utilized a modified protocol of Pfeffer et al. (2003). PCR primers specific to each arm of the vMir-predicted pre-miRNAs were used to individually map the 5′ and 3′ ends of the 5p and 3p miRNAs encoded by JCV and BKV. The results from these mapping studies showed some but not perfect nucleotide identity between the SV40 and BKV and JCV miRNAs. Importantly, both the 5p and 3p miRNAs from JCV and BKV have several nucleotide differences in their seed sequences compared to the SV40 miRNAs (Figure 2.1D), suggesting different binding sites in their respective targets.

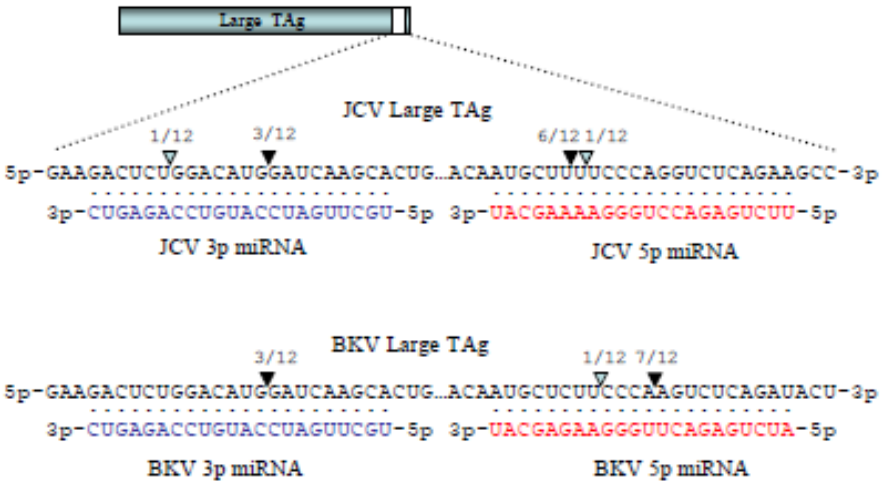

Figure 2.2 5′ RACE analysis demonstrates that each miRNA is active at directing the cleavage of the early mRNAs. A modified RACE protocol was utilized to screen for miRNA-mediated cleavage fragments. Portions of the early RNAs corresponding to the coding regions for the large TAgs are shown 5′ to 3′. The positions of the predominant 5′ ends of the cleavage fragments are denoted with black arrowheads, and the numbers of corresponding amplicons are shown. Gray arrowheads indicate the positions of the 5′ ends of less-common amplicons. miRNAs from the complementary strand are shown 3′ to 5′.

2.2.2 JCV and BKV miRNAs direct the cleavage of the early viral transcripts.

Since the 5p and 3p miRNAs are perfectly complementary to early mRNAs, we next examined whether they direct the cleavage of the early RNAs in a small interfering RNA-like fashion. We used a modified 5′ RACE analysis (Llave et al., 2002; Mansfield et al., 2004; Yekta et al., 2004) to look for miRNA-mediated cleavage fragments. This analysis showed that, indeed, all four miRNAs direct the cleavage of their respective complementary early RNAs. The 5′ ends of the majority of the amplicons we sequenced (22 of 24) were complementary to the middle of an miRNA we identified (Figure 2. 2). Notably, for both JCV and BKV, more cleavage fragments mapped to the middle of the 5p miRNA than to the 3p miRNA. This result is consistent with our observation that the 5p miRNAs of both JCV and BKV are more abundant than the 3p miRNAs (Figure 2.1B and C). To our knowledge, the polyomaviruses represent the only example in which different miRNAs, derived from both arms of a single pre-miRNA precursor, are active on the same molecular targets (the early mRNAs). These results show that multiple polyomaviruses encode miRNAs to autoregulate mRNA levels.

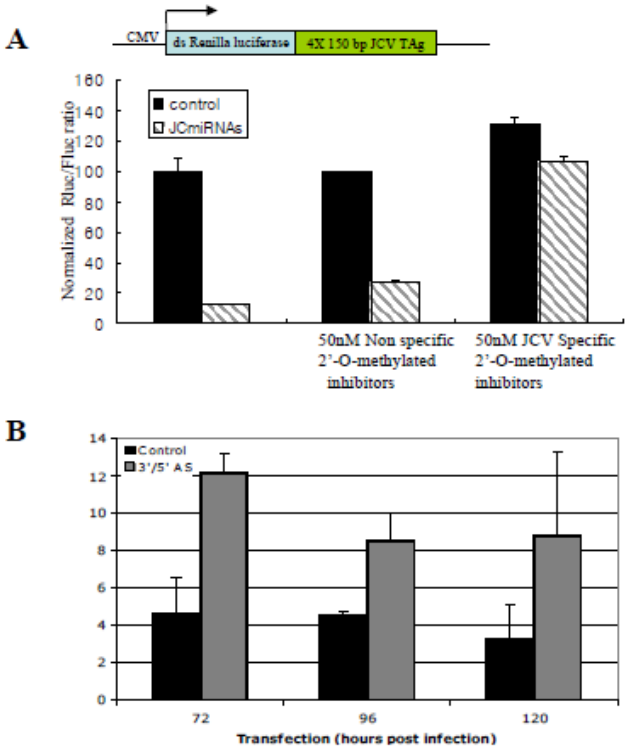

Figure 2.3 The JCV miRNAs expressed late during infection downregulate early protein levels. (A) A diagram of a Renilla luciferase reporter containing the region of the early genome complementary to the JCV miRNAs is shown (top). The graph shows luciferase levels from cells transfected with the reporter plus a plasmid expressing the JCV miRNAs (JCmiRNAs) or a negative control plasmid. The Renilla luciferase (Rluc) values were normalized to the values for firefly luciferase (Fluc; cotransfection control). CMV, cytomegalovirus; ds Renilla luciferase, double-stranded Renilla luciferase cDNA; 4X 150 bp JCV TAg, four copies of the JCV TAg construct. (B) A quantitative immunoblot analysis of the large TAg proteins shows that transfection with specific inhibitors (3′/5′ AS) of the JCV miRNAs increased early protein levels during infection. Results from transfection with an irrelevant control oligonucleotide are also shown. The data presented are the averages of results from three independent experiments, plotted as percentages of the protein standards loaded onto each gel.

2.2.3 JCV miRNAs downregulate large-TAg expression late during infection.

We predicted that the cleavage of the early mRNAs late during infection should result in a decrease in early protein levels. To test this hypothesis, we designed two different 2′-O-methylated antisense oligonucleotides complementary to either the 5p or 3p JCV miRNA by using a strategy similar to that of Vermeulen et al. (2007). To quantitatively measurelarge TAg that is complementary to the JCV miRNAs (Figure 2.3A). As expected, the cotransfection of cells with the reporter and a plasmid expressing the JCV pre-miRNA their inhibitory activities, we developed a luciferase-based reporter assay. The reporter construct contained four iterative copies of a region of the genome encoding the JCV resulted in a dramatic decrease in luciferase levels, while no decrease was detected with a control reporter (lacking the JCV complementary region) (Figure 2.3A and data not shown). However, when cells were cotransfected with both the 5p and 3p inhibitors and the reporter and JCV miRNA constructs, almost no decrease in luciferase activity was observed. Transfection with an irrelevant control oligonucleotide had little effect in this assay. These results demonstrate that the JCV miRNAs can negatively regulate the expression of reporters containing 150 nucleotides of complementary early gene transcripts and that our antisense inhibitors blocked JCV miRNA-mediated mRNA cleavage. When we infected cells with JCV and then transfected them with the pooled 5p and 3p antisense inhibitors, a highly reproducible, albeit modest, increase in large-TAg protein levels was observable at late times postinfection (Figure 2.3B), when the miRNAs are made. These results demonstrate that the JCV miRNAs downregulate early protein

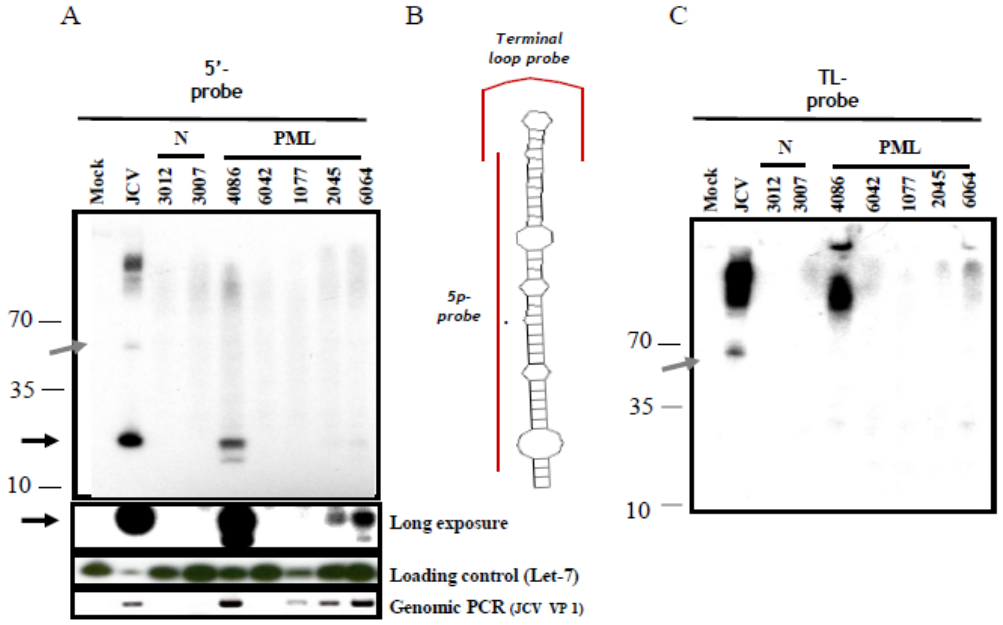

Figure 2.4 JCV miRNAs are detected in the brain tissue of PML patients. Total RNA was harvested from sections of brain tissue from patients diagnosed with PML. Northern blot analyses with probes specific for the JCV 5p and the loading-control let-7 miRNAs are shown. Northern blotting with a probe specific for the loop region of pre-miRNA showed that the JCV miRNA bands detected in brain tissue were not degradation fragments. (A) Full-length blot showing the detection of the JCV miRNA and pre-miRNA with the 5p probe. (B) Diagram of probes used. (C) Full-length blot from panel A, stripped and reprobed with the terminal loop (TL)-probe. Note that the control loop probe and the 5p probe both detect the pre-miRNA but that only the 5p probe detects bands at ~22 nucleotides. This pattern rules out the possibility that degradation may account for the miRNA-specific bands detected. The miRNAs detected with the 5p probe are indicated with black arrows. The band corresponding to the pre-miRNA is indicated with a gray arrow. Numbers to the left of the blots indicate sizes in nucleotides. N, normal tissue samples.

levels and that the antisense inhibitors we designed were effective at blocking, at least partially, the functions of both JCV miRNAs.

2.2.4 JCV miRNAs are readily detectable in brain tissues of PML patients.

Most viral miRNAs have been identified by the infection of cultured cells in vitro. To examine whether the JCV miRNAs are made during infection in vivo, postmortem tissue samples from the brains of patients diagnosed with PML were obtained. We harvested total RNA and probed for the more abundant 5p JCV miRNA. Four of the five PML samples were positive for viral DNA, as assayed by PCR. Notably, the three samples with the highest levels of viral DNA (samples 4086, 2045, and 6064) scored positive for the JCV miRNA. One sample (4086) scored almost as high as the in vitro-infected cells (Figure 2.4A and C, compare lanes 4086 to lanes JCV). The miRNA-specific band detected was unlikely to represent a degradation fragment, since a control probe for the loop region of the pre-miRNA (which corresponds to a sequence flanking the miRNAs and therefore should detect the pre-miRNA band but not the processed, stable ~22-nucleotide miRNA band) (Figure 2.4B) detected the pre-miRNA band without detecting any signal around ~22 nucleotides (Figure 2.4C). This result strongly suggests that there was no general enrichment of our samples with nonspecific cleavage fragments in the ~22-nucleotide size range that may account for the miRNA-specific signal we detected. Due to the challenges of isolating PML lesions away from normal brain tissue, we believe that most of our PML lesion samples were contaminated with various amounts of normal, uninfected tissue. Thus, these results likely underrepresent

the actual abundance of this miRNA in PML lesions. These data suggest that a majority of PML lesions in which JCV is actively replicating contain high enough levels of the JCV miRNA to be detected by Northern blot analysis. To our knowledge, this is the first report of a viral miRNA being detected in vivo in noncultured human tissue samples.

2.3 DISCUSSION

Human polyomaviruses cause serious disease in immunocompromised patients, and effective therapies are lacking. We set out to explore whether the human polyomaviruses JCV and BKV encode miRNAs, a class of small RNA regulatory molecules that bind to and (typically) inactivate target mRNAs. Here, we have conclusively shown that homologous pre-miRNAs expressed by JCV and BKV show striking similarities to a pre-miRNA encoded by the related monkey polyomavirus SV40. JCV, BKV, and SV40 encode homologous pre-miRNA hairpins, both arms of which are processed into mature miRNAs. Despite being only ~65% identical (5p miRNAs are ~55% identical, and 3p miRNAs are ~75% identical), all three pre-miRNAs share several atypical properties in terms of processing and abundance. Significantly, all produce two different miRNAs, both of which regulate early gene expression at late times of infection. To our knowledge, the pre-miRNAs encoded by the polyomaviruses are the only pre-miRNAs known to produce miRNAs from both strands of the precursor hairpin that are active on the same target (the early RNAs). Few viral miRNAs identified so far are evolutionarily conserved, and it is unclear if any have conserved functions. Notably,

the seed region (the 5′ nucleotides of an miRNA, from positions 2 to 8) (Figure 2.1D), which plays a crucial role in determining miRNA target specificity (Lai, 2002; Lim et al., 2005), is not conserved between SV40 miRNAs and the human polyomavirus miRNAs. This suggests that a major function of the polyomaviral miRNAs is their conserved abilities to regulate their corresponding early RNAs, which necessarily have perfect complementarity to the miRNAs (including the seed region) generated from the opposite strand.

At least one of these miRNAs (from JCV) is expressed at robust levels during pathological infection in humans. Currently, there are no effective therapies against polyomaviral infection. As the numbers of immunosuppressed transplant and AIDS patients grow, so does the need for antipolyomaviral drugs. We have previously shown in in vitro assays that miRNA-mediated autoregulation of early gene expression can reduce cytotoxic T-cell-mediated lysis of SV40-infected cells. These data suggest a model in which the primate polyomaviral miRNAs may function to evade the immune response (Sullivan et al., 2006; Vermulen et al., 2007). The implications of this model, combined with the recent successes in delivering anti-miRNA inhibitors directly into the brains of mice (Krutzfeldt et al., 2005; Krutzfeldt et al.,2007), suggest that a similar approach directed against the JCV miRNAs may provide a treatment strategy for PML.

In summary, we have demonstrated that multiple members of the polyomavirus family encode a pre-miRNA with a function that has been conserved throughout millions of

years of evolution. This finding suggests the likelihood that some miRNAs in other virus families may also have conserved functions.

2.4 MATERIALS AND METHODS

2.4.1 Computational prediction of viral miRNA precursors and miRNA cloning.

We used a new version of vMir (Grundoff et al., 2006) with a vMir cutoff score of 175 to obtain candidate pre-miRNAs from the genomes of BKV and JCV (the genome accession numbers are as follows: BKV, NC_001538, and JCV, NC_001699). For the purpose of JCV miRNA cloning, we made an miRNA library from total RNA harvested from SVG cells infected with JCV. The library was generated utilizing a modified protocol of Pfeffer et al. (Pfeffer and Voineet, 2006). Briefly, total RNA was run on a denaturing 15% polyacrylamide gel, and small RNAs (approximately 10 to 40 nucleotides in length) were isolated. Small RNAs were eluted overnight from the gel slurries and ethanol precipitated using the Megaclear kit (Ambion, Austin, TX) according to the manufacturer's suggested protocol for harvesting small RNAs. Small RNAs were ligated to a preadenylated 3′ linker (5′-CTGTAGGCACCATCAATCCddC-3′, where “dd” is deoxy ribose, which blocks the 3′ end) with T4 RNA ligase (Ambion, Austin, TX). Ligation products were gel purified, eluted, and ligated to the 5′ oligonucleotide (nonpreadenylated linker) (5′-CAGTTGATCAGAGCCCArGrGrG-3′, where “r” is ribose). Next, cDNAs were synthesized using reverse transcriptase (Ambion, Austin, TX), and the resulting products were cloned using a TA cloning kit (Invitrogen, Carlsbad, CA). Different primers specific to either the 5′ or 3′ portions of the predicted JCV

miRNAs, combined with linker-specific primers, were utilized for PCR amplification. To amplify and map the JCV miRNAs, we performed nested PCRs. Resulting products were sequenced to define the 5′ and 3′ ends of the JCV 5′-arm (5p) and 3′-arm (3p) miRNAs. The PCR primers used were as follows: 5′ linker forward primer, GTTGATCAGAGCCCAGGG; 3′ linker backward primer, ATTGATGGTGCCTACAG; JCV 5p forward primer, TCTGAGA CCTGGG AAA; JCV 5p backward primer, AATCACAATGCTTTTCC; JCV 3p outer forward primer, AGAGCCCAGGGTGCTTG; JCV 3p inner forward primer, TGCTTGATCCATGTCCAGA; JCV 3p outer backward primer, GATGGTGCCTACAGGACTCT; and JCV 3p inner backward primer, GACTCTGGACATGGATCAAG.

2.4.2 Cell culture, virus infection, and PML brain tissues.

The human glial cell line U87 and the green monkey kidney cell line Vero were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were routinely tested to ensure that they were free of mycoplasma contamination. The cells were infected with 512 hemagglutination units of JCV (Mad-1/SVE delta strain) or BKV (Dunlop strain) for 1 h at 37°C in reduced (2%) serum. Posthumous clinical brain tissue samples were obtained from the National Neurological AIDS Bank (NNAB; Los Angeles, CA). All the samples came from pathologically normal brain tissues or the brains of patients diagnosed microscopically with PML. The diagnosis was made in all

cases based on the presence of typical histological features of PML, along with the clinical history. Samples were also confirmed to be JCV positive via PCR analysis utilizing primers that amplified a region of the genome corresponding to a portion of JCV VP1. The PCR primers used were as follows: VP1 gene forward primer, CCCAGCAGTGGAGAGGACT, and VP1 gene backward primer, CAGCATTTTTGTCTGCAACTG.

2.4.3 RNA isolation and miRNA Northern blotting.

Total RNA was harvested from cultured cells infected with BKV or JCV or from solid brain tissue samples by using RNAbee according to the manufacturer's (TelTest Labs) directions. RNA was run on a Tris-borate-EDTA-urea-15% polyacrylamide gel. The gel was transferred onto a Hybond H+ membrane and probed for candidate miRNAs as described previously (Grundhoff et al., 2006). The blot was probed with radiolabeled oligonucleotides generated with P^{32}-labeled radioactive gamma phosphate and T4 polynucleotide kinase (U.S. Biologicals, Cleveland, OH). The blot was first probed with the 5p probe, stripped with boiling hot stripping buffer (0.1% sodium dodecyl sulfate in double-distilled water), and then probed with the 3p probe, the internal-loop probe, or the control probe (hsa-let-7a). The probe sequences used were as follows: JCV 5p probe, CAATCACAATGCTTTTCCCAGGTCTCAGAAGCCTCT; JCV 3p probe, CAGAAGACTCTGGACATGGATCAAGCACTGAATCA; JCV internal-loop probe, AGCACTGAATCACAATCACAAT; BKV 5p probe, CAATCACAATGCTCTTCCCAAGTCTCAGATACTTCA; BKV 3p probe,

ACTGAAGACTCTGGACATGGATCAAGCACTGAATCC; and hsa-let-7a probe, TGAGGTAGTAGGTTGTATAGTT.

2.4.4 Modified 5′ RACE for the detection of JCV and BKV early gene fragments.

mRNA cleavage fragments from JCV- and BKV-infected cells were identified utilizing a modified rapid amplification of cDNA ends (RACE) protocol (Llave et al., 2002) with a FirstChoice RNA-ligase-mediated-RACE kit (Ambion, Austin, TX). A 5′ RACE adaptor was ligated to total RNA extracted from infected cells. Reverse transcription followed by nested PCRs was performed using the 5′ RACE adapter primers and JCV- or BKV-specific primers. PCR products were cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). Twelve clones each from JCV and BKV were sequenced. The primer sequences used for the PCRs were as follows: 5′ adaptor, GCGAGCACAGAATTAATACGACTCACTATAGG$(T)_{12}$VN; 5′ RACE outer primer, GCGAGCACAGAATTAATACGACT; 5′ RACE inner primer, CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG; JCV outer primer, GTAATA TGCAGTACATTTTAAT; JCV inner primer, TACATTTTAATAAAGTATAAC; BKV outer primer, GTACATATACATTTAATAAATGC; and BKV inner primer, ATTTAATAAATGCTGCTTTTG.

2.4.5 DNA constructs, transfection, and luciferase assay.

All DNA constructs were confirmed by sequence analysis. The plasmid pCDNA3.1JCV miRNA, which expresses JCV miRNA, was generated by cloning the PCR-amplified

portion of the JCV genome into the BamHI/XhoI sites of the pCDNA3.1puro expression vector (Sullivan and Ganem, 2005). A plasmid containing the entire JCV genome (kindly provided by Mike Imperiale, University of Michigan) served as the template to amplify the region of the JCV genome corresponding to the JCV pre-miRNA, as well as ~1 kb of flanking regions. The primers used were as follows: JCV miRNA construction forward primer, CCTTGGATCCCTTCTTATAAGAGGAGGAGTAG, and JCV miRNA construction backward primer, GAGACTCGAGTTGGAAACCAAGTGTGAGGATG.

pCDNA3.1dsRluc and pCDNA3.1dsFFluc, expressing destabilized *Renilla* luciferase and firefly luciferase cDNAs, were constructed by PCR amplification of a luciferase gene insert generated from the plasmid templates pGL4.84hRlucCP/Puro (Promega; catalog no. E7521) and pMSCV-dsLuc2cp (a kind gift from Andrei Goga, University of California, San Francisco). PCR products were cloned into the KpnI/XhoI sites of the pCDNA3.1puro expression vector. The primers used were as follows: *Renilla* luciferase gene forward primer, ATTGGTACCATGGCTTCCAAGGTGTACGACCC; firefly luciferase gene forward primer, GCTGGTACCATGGAAGATGCCAAAAACATTAAG; and shared firefly and *Renilla* luciferase gene backward primer, AATCTCGAGTTAGACGTTGATCCTGGCGCTGGC.

A concatemerized region of the JCV genome corresponding to a portion of the large T antigen (TAg) was subcloned into the 3′ untranslated region of pCDNA3.1dsRluc. This 150-bp region of pCDNA3.1dsRluc contains sequences complementary to those of the JCV 5p and 3p miRNAs, as well as additional flanking regions. PCR utilizing primers

that contain the nonpalindromic BanI restriction enzyme site was used to generate concatemers. The primers used were as follows: JCV TAg gene forward primer, GAAGGCACCAGACCCATTCTTGACTTTCCT, and JCV TAg gene backward primer, GCACCACGGACAGATGTGAAAGTGCAGTTT.

PCR products were digested with BanI, purified, and ligated with T4 DNA ligase (New England Biolabs, Inc., Beverley, MA). *Taq* polymerase (New England Biolabs, Inc., Beverley, MA) was used to fill in the overhanging nucleotides, and the resulting products were cloned using the TA cloning kit. Concatemers of the four copies of the JCV TAg construct (pCR2.1JCVTAg) were selected by restriction digest analysis and subcloned into the XhoI/XbaI sites of pCDNA3.1dsRluc vectors. PCR was used to generate inserts to subclone from the TA vector pCR2.1JCVTAg into pcDNA3.1. The primers used were as follows: JCV TAg concatemer forward primer, ATGCTCGAGCGGCCGCCAGTGTGATGGATA, and JCV TAg concatemer backward primer, GCATCTAGAGTAACGGCCGCCAGTGTGCTG.

293 cells were obtained from the ATCC and cultured in Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum. 293 cells were plated into 12-well dishes and transfected using the Lipofectamine 2000 reagent. Cells were transfected with the pCDNA3.1dsRlucJCVTAg plasmid, as well as pcDNA3.1dsFFluc as a transfection control, along with the pCDNA3.1 JCV miRNA expression vector. Transfections were conducted in the presence of a cocktail of 50 nM 2′-O-methylated inhibitors specific for the 5p and 3p miRNAs (see below) or an irrelevant control

oligonucleotide. Cells were harvested 48 h after transfection and analyzed via a dual luciferase assay system (Dual-Glo luciferase assay system; catalog no. E2980) according to the recommendations of the manufacturer (Promega). The luciferase assay results were read on a Luminoskan Ascent luminometer (Thermo Electronic Corporation, Milford, MA). Results are presented with the *Renilla* luciferase levels normalized by the firefly luciferase levels.

2.4.6 Antisense oligonucleotides.

The modified antisense oligonucleotides were Dharmacon products synthesized by Thermo Fisher Scientific (Lafayette, CO). The antisense oligonucleotides were designed by a method similar to that described by Vermeulen et al. and contained 2′-O-methylated nucleotides (Vermeulen et al., 2007). The primers used were as follows: irrelevant control antisense primer, AGAAGAGAGAAAUCUCUUCUUGGCCACUCGGGGGGACAACACUAAUCGCC AACAGACAUCUUCUCUUUCGAGAGAAGA; JCV 3p miRNA antisense primer, AGAAGAGAGAAAUCUCUUCUCAGAAGACUCUGGACAUGGAUCAAGCACUG AAUCACAAUCUUCUCUUUCGAGAGAAGA; and JCV 5p miRNA antisense primer, AGAAGAGAGAAAUCUCUUCUCUGAAUCACAAUCACAAUGCUUUUCCCAGG UCUCAUCUUCUCUUUCGAGAGAAGA.

2.4.7 Transfection with antisense oligonucleotides and Western blot analysis of JCV-infected cells.

Control and infected cells were transfected every 24 h, throughout the duration of infection, with either 50 nM irrelevant 2′-O-methylated oligonucleotide (control) or 50 nM (each) 2′-O-methylated antisense oligonucleotides directed at the JCV 3p and 5p miRNA sequences. Transfections were conducted using Lipofectamine 2000 according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA). All samples were collected by scraping at 6 days postinfection and lysed for 3 h in 50 μl of 1× radioimmunoprecipitation assay buffer on ice. Protein concentrations were quantified by a Bradford assay, and results were read on an Eppendorf biophotometer. Fifty micrograms of protein from each sample was analyzed by electrophoresis on a 4 to 15% Tris-HCl gel for 1.5 h at 30 mA, and the proteins were transferred overnight onto nitrocellulose membranes at 4°C and 20 V. The membranes were then probed with an anti-SV40 TAg monoclonal antibody, diluted 1:1,000, that is known to cross-react with JCV TAg. Bound primary antibody was detected with goat anti-mouse horseradish peroxidase-conjugated secondary antibody diluted 1:20,000. The blots were then developed using the Rodeo sensitive Western blotting kit (U.S. Biologicals, Cleveland, OH). Bands were visualized by chemiluminescence on a ChemiDoc XRS system (Bio-Rad, Hercules, CA). Levels of TAg expressed in each sample were quantified using Bio-Rad Quantity One gel analysis software, and results were calculated relative to values for a loading control as percentages of the known amount of protein loaded in the standardized controls according to the manufacturer's directions. The results are displayed as percentages of TAg relative to multiple protein standards (Kaleidoscope; Bio-Rad).

Chapter 3: Merkel cell polyomavirus encodes a microRNA with the ability to autoregulate viral gene expression

3.1 INTRODUCTION

microRNAs (miRNAs) are post-transcriptional regulators of gene expression that play a role in numerous and diverse cellular processes including viral infection (reviewed in (Gottwein and Cullen, 2008; Grey, Hook, and Nelson, 2008; Nair and Zavolan, 2006; Pfeffer, 2008; Samols and Renne, 2006; Sarnow et al., 2006; Sullivan, 2008; Sullivan and Ganem, 2005)). Over 120 viral miRNAs have been described from divergent families of DNA viruses. A functional understanding of the majority of viral miRNAs remains incomplete or unknown, however recent studies clearly demonstrate that some viral miRNAs will regulate host gene expression, viral gene expression, or both. Viral miRNAs that regulate host transcripts with relevance to tumorigenesis and immune evasion have been described. In addition, diverse viruses, including an ascovirus (Hussain, Taft, and Asgari, 2008), and several members of the Herpesviridae and Polyomaviridae have been shown to encode miRNAs that autoregulate viral gene expression (Gottwein and Cullen, 2008; Pfeffer, 2008). It has been hypothesized that viral miRNA-mediated autoregulation of gene expression contributes to regulation of the virus lifecycle including the maintenance of latency and activation of lytic replication (Umbach et al., 2008) - the proper balance of which is key to immune evasion strategies. Therefore, proper functioning of autoregulatory miRNAs likely profoundly affects the

fitness of some viruses during natural infection *in vivo*. Thus, combined with the recent successes in rational-designed anti-miRNA inhibitors (Elmen et al., 2008; Krutzfeldt et al., 2005), these observations suggest viral miRNAs might make worthy anti-viral drug targets. Members of the Polyomaviridae include the model laboratory tumor viruses murine polyomavirus (MuPyV) and Simian Virus 40 (SV40), and the human pathogens BK virus (BKV) and JC virus (JCV). We have previously shown that multiple members of the Polyomaviridae, including SV40, JCV, BKV, and MuPyV encode miRNAs that autoregulate early gene expression at late times of infection (Seo et al., 2008; Sullivan et al., 2006; Sullivan et al., 2005; Sullivan et al., 2008). Recently, three new human polyomaviruses have been described, including KI virus (KIV), WU virus (WUV) and Merkel cell polyomavirus (MCV) (Allander et al., 2007; Feng et al., 2008; Gaynor et al., 2007). All three were discovered using new molecular-based technologies. It seems clear from these and other related technologies that we are in for an avalanche of new virus discoveries. Many of these viruses are being discovered faster than *in vitro* culture systems can be developed. Here we present a method for identifying viral-encoded miRNAs that does not require an experimental infectious system. We show that MCV, a likely etiologic agent of human cancer, encodes a miRNA on the late strand that (similar to other reported Polyomaviral miRNAs) has the potential to autoregulate early gene expression. These results further emphasize an important role for miRNAs in Polyomaviral biology and suggest a possible target for future therapeutic strategies.

3.2 RESULTS

We set out to develop an experimental strategy to identify viral-encoded miRNAs that does not require abundant amounts of infected material. We chose to examine whether MCV encoded a miRNA for the following reasons: First, MCV was only recently discovered and so far no experimental infectious systems have been reported. Second, MCV is linked to human cancer and is likely a bona fide human pathogen, suggesting a possible need to develop anti-viral therapeutic targets. Third, because we have previously shown divergent members of the polyomavirus family (including the SV40-like polyomaviruses and MuPyV) encode miRNAs to autoregulate early gene expression; it seemed probable that other members of the family, including MCV, would also encode miRNAs of similar function. Our strategy is to computationally predict likely candidate pre-microRNAs (pre-miRNAs) and then to clone them, including flanking sequence, into a heterologous expression vector under control of the cytomegalovirus major early promoter (CMV). These plasmids are then transiently transfected and screened for bona fide miRNA expression using several criteria including: Northern blot analysis, cloning and sequencing, and activity in chimeric target reporter assays. We ran the sequence of both published MCV isolates in the viral miRNA prediction algorithm Vmir (Grundhoff, Sullivan, and Ganem, 2006; Sullivan and Grundhoff, 2007; Sullivan et al., 2005). A relatively stringent arbitrary cutoff score of 200 was used and we focused on transcripts of the late orientation where all other Polyomaviral miRNAs have so far been identified. Only two candidates met these criteria (Figure 3.1A). One candidate, MR62 was found near the origin of replication and was predicted for both the MCC339 and

MCC350 isolate genomes. However, this candidate was discarded because this region of the genome produced false positive candidates from several other members of the Polyomaviridae (CSS, unpublished observations). The other candidate, MR17, was the top scoring Vmir prediction for MCC339. However, due to three nucleotide substitutions in the pre-miRNA hairpin region (Figure 3.1B), MR17 was ranked only the sixth best candidate for MCC350. We nonetheless pursued candidate MR17 (although it shared no sequence identity with any other known pre-miRNAs of viral or host origin), because it did however, map to a region of the genome we have previously shown encodes a pre-miRNA in MuPyV (Sullivan et al., 2008).

A portion of the genome including candidate MR17 and flanking regions was cloned from either the MCC339 or MCC350 isolates into an expression vector. The resulting plasmids were then transfected into 293T cells. Total RNA was harvested and Northern blot analysis was conducted with the probes described in Figure 3.2A. The 5p probe detected 2 bands from RNA harvested from cells transfected with either the MCC339 or MCC350 expression vector. The slower migrating band is approximately 65 nucleotides (consistent with it being a pre-miRNA), and the faster migrating band is approximately 22 nucleotides- exactly where a *bona fide* miRNA should migrate. The 3p probe detected the slower ~65 nucleotide band and low amounts of a ~22 nucleotide band. Importantly, no specific bands were detected from RNA harvested from cells transfected with a negative control vector in which a 300 nucleotide portion of MCC350 (containing the pre-miRNA and flanking regions) was deleted. In addition, a control

probe directed against the terminal "loop" portion of the predicted pre-miRNA hairpin, which should not be stabilized by the downstream miRNA processing and effector machinery, only recognized the ~65 nucleotide band. These results rule out non-specific RNA degradation as a source of the 22 nucleotide band detected with the 5p probe. Combined, these results strongly suggest that multiple isolates of MCV encode a pre-miRNA in the late orientation, of which both the 5p and 3p arms of the hairpin are processed into miRNAs. In accordance with the established miRBase precedence (Griffiths-Jones, 2006), we have named these miRNAs "MCV-mir-M1 5p" and "MCV-mir-M1 3p".

To map the derivative miRNAs with more precision we generated a small RNA library from cells expressing the MCC350 miRNAs. Small RNAs were gel purified and ligated to linkers, and reverse transcription was performed to generate a cDNA library. PCR was then conducted with primers specific to each arm of the V-mir predicted pre-miRNA hairpin. Using this strategy we were able to individually map the 3' and 5' ends of both the 3p and 5p miRNAs. Notably, the miRNA sequences we mapped from the MCC350 isolate are completely conserved with the MCC339 isolate. (Figure 3.1B).

We next sought to determine if MCV-mir-M1 was a functionally active miRNA. miRNAs are initially transcribed as long precursor molecules that contain a characteristic ~80-100 nucleotide hairpin secondary structure. This hairpin is recognized and excised by the nuclear microprocessor multi-protein complex resulting in an ~53-80 nucleotide precursor miRNA (pre-miRNA) (reviewed in (Gottwein and Cullen, 2008)). The pre-

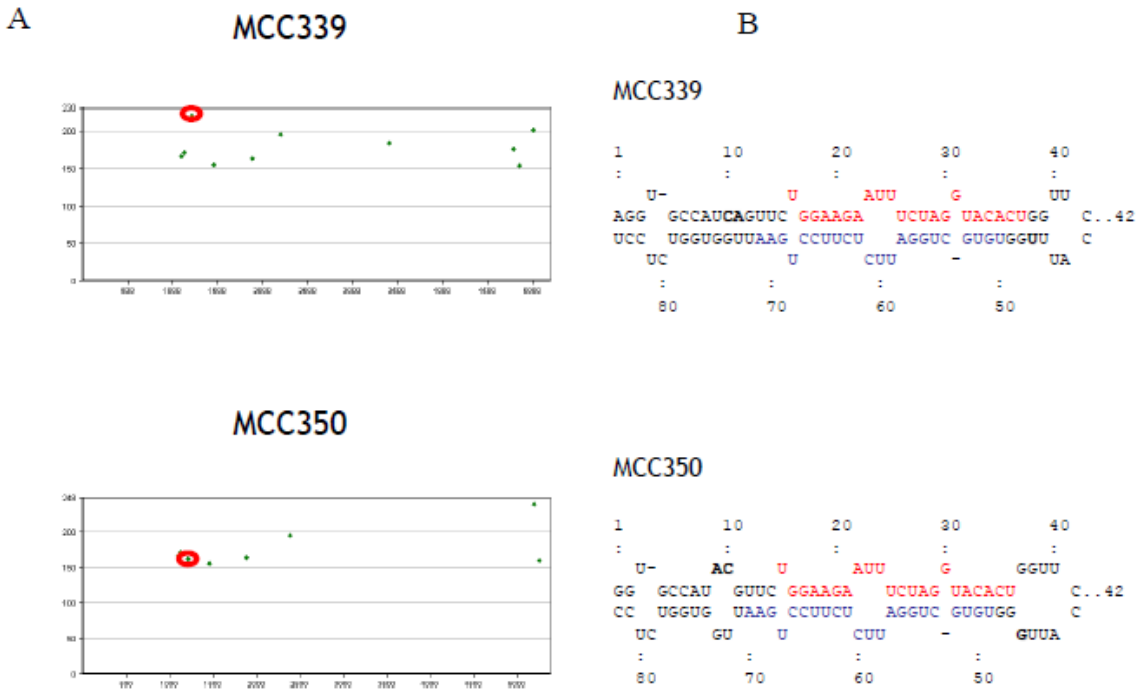

Figure 3.1 Vmir predictions for MCV pre-miRNAs. **(A)** Candidate pre-miRNAs are indicated for both the MCC 339 and MCC 350 isolates (diamonds). The candidate that scored positive in both MCC339 and MCC350 (referred to as MR17 in the text) is marked with a red circle. **(B)** The secondary structure predictions for MCV precursors are shown. The polymorphic sites between MCC 350 and MCC 339 are shown (bold). The fine-mapped sequences of the 5p (red) and 3p miRNAs (blue) are indicated.

"arm" of the pre-miRNA hairpin is incorporated in a stable manner into the multi-protein RNA Induced Silencing Complex (RISC). RISC-bound miRNAs can then bind to target miRNA is further processed by the cytoplasmic, RNAse III-like endonuclease Dicer into a transient double-stranded ~ 22 nucleotide intermediate. Eventually, a portion of a single mRNAs with perfect or imperfect complementarity, typically resulting in cleavage or translational repression of the targeted mRNA. We have previously shown that multiple members of the Polyomaviridae encode miRNAs that are active within RISC to direct the cleavage of the complementary early strand RNAs late during infection (Seo et al., 2008; Sullivan, 2008; Sullivan et al., 2006; Sullivan et al., 2005; Sullivan et al., 2008). Given that MCV-mir-M1 is encoded on the late strand, antisense to the early transcripts (Figure 3.3A&B), we predicted that it would direct the cleavage of the early transcripts. To test this hypothesis, we engineered a chimeric luciferase reporter construct, in which a 300 nucleotide portion of the early transcripts (including the MCV-mir-M1 complementary and flanking regions) was cloned into the 3' UTR. Co-transfection of this reporter with either the MCC339 or MCC350 MCV-mir-M1 expression plasmids results in a dramatic reduction in luciferase activity (Figure 3.3C, left panel). Co-transfections of vector alone, an irrelevant miRNA (JCV), or a vector encoding MCC350 with the miRNA region deleted had no effect on this reporter (Figure 3.3C, left panel) arguing that the effect we observe is specific to the MCV miRNAs. Importantly, no effect of the MCV miRNAs was detected when they were co-transfected with a control reporter plasmid that lacked complementary sites to MCV-mir-M1 (Figure 3.3C, right panel). From these results, two inferences can be made. First, MCV-mir-M1 is fully active within RISC, lending further

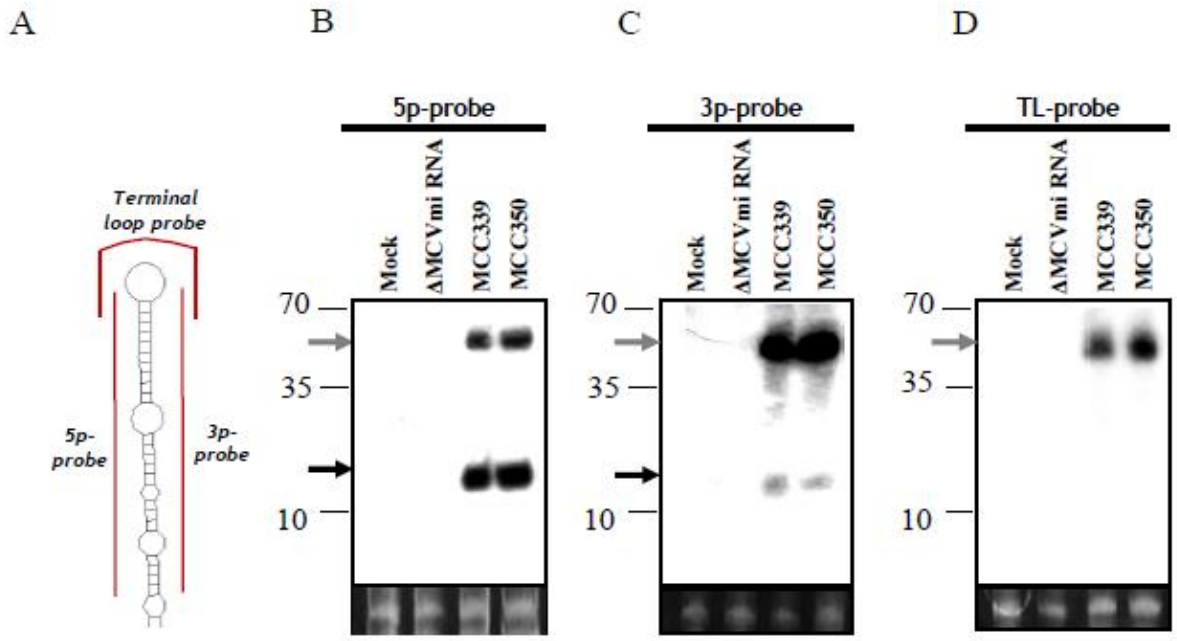

Figure 3.2 The Merkel cell poloymavirus isolates MCC 339 and MCV 350 encodes miRNAs. (A) Diagram of probes used. Each sample was assayed with a probe that detects the 5p or 3p arm or terminal loop portion of the pre-miRNA. (B-D) Northern blot analysis for the MCV miRNAs. RNA harvested from cells transfected with the mock, negative control **Δ** MCV miRNA, the MCC339 or MCC350 miRNA expression vectors was analyzed with the indicated probes. Ethidium bromide staining of the low molecular weight RNA is shown as a loading control. Bands corresponding to the 5p and 3p miRNAs (black arrow) or the pre-miRNA (gray arrow) are indicated.

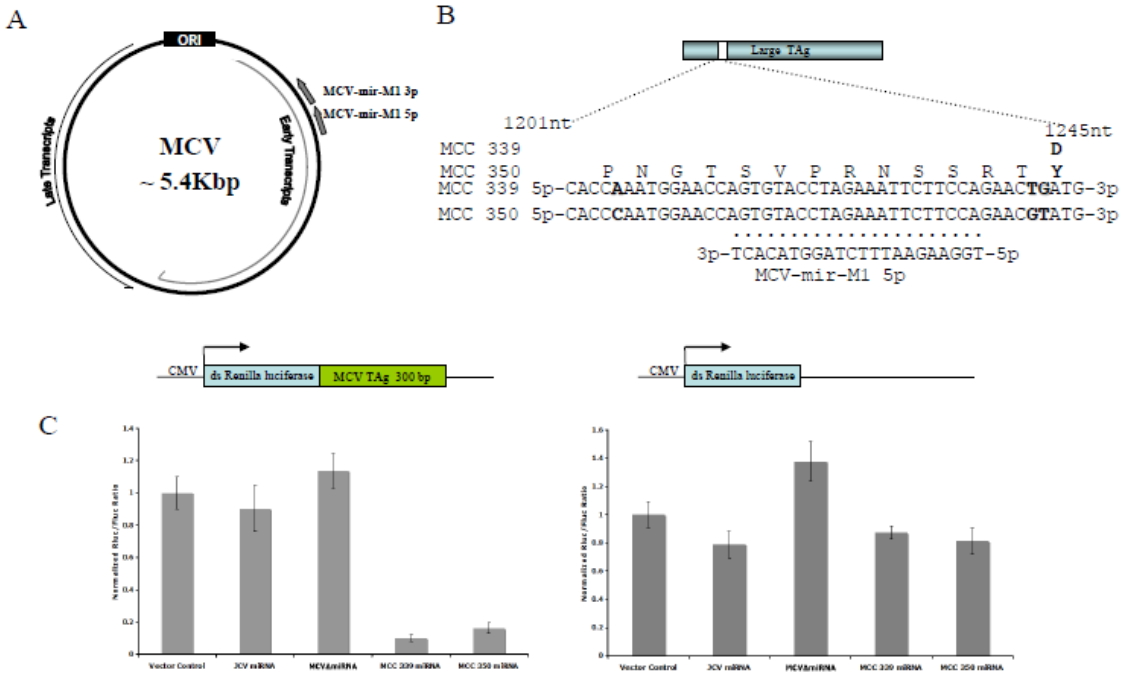

Figure 3.3 The MCV miRNAs downregulate early transcripts. (A) Map of MCV genomic organization with the location of the 5p and 3p miRNA indicated with gray arrows. (B) Alignment of the MCV early transcripts (5' - 3') with the complementary 5p miRNA indicated (3' - 5'). Shown are the derived amino acids of MCV Large T Antigen that correspond to this region. (C) Diagrams of the MCV miRNA reporter construct (top, left) and control reporter (top, right) are shown. The vectors consist of a Renilla luciferase reporter with or without a single copy of a 300 nucleotide region of the Large T antigen early transcript (complementary to the MCV miRNA) cloned into the 3'UTR. Cells were transfected with either reporter plus a plasmid expressing vector alone, the MCV339 miRNA, the MCV350 miRNA, the JCV miRNA, or **Δ** MCV miRNA (a negative control MCV plasmid in which the pre-miRNA region has been deleted). Firefly luciferase was used as a transfection control and Renilla luciferase levels are plotted normalized relative to firefly luciferase levels.

support as to the authenticity of this miRNA. Second, MCV-mir-M1 functions to direct cleavage of the early RNAs during MCV infection.

3.3 DISCUSSION

The post-genomic era has ushered in several new virus discovery techniques that will likely provide a windfall of new pathogens in the near future. Many of these viruses will not have a readily available experimental system making developing and testing anti-viral drugs a challenge. We propose that one useful strategy will be to identify virus-encoded miRNAs and utilize existing successful antisense anti-miRNA drug strategies (Elmen et al., 2008; Krutzfeldt et al., 2005) to develop potential therapeutics. A first step to realizing this goal is to develop methods to identify miRNAs encoded by such pathogens. In this work, we describe such a method and prove its utility on the recently described polyomavirus MCV. We show that two different strains of MCV encode the miRNA despite having a difference of 3 nucleotide substitutions in the pre-miRNA hairpin. Interestingly, these substitutions result in 2 non-conservative amino acid substitutions in the Large T antigen protein that is encoded on the early strand (Figure 3.3B). However, these changes do not significantly alter the predicted pre-miRNA hairpin secondary structures on the late strand (Figure 3.1B) and the pre-miRNAs from both the MCC339 and MCC350 isolates have similar processing efficiencies (Figure 3.2). Furthermore, the derivative miRNAs from both the MCC350 and MCC339 isolates have similar activities in directing the cleavage of the early transcript reporter (Figure 3.3C).

These results suggest evolutionary pressure exists for MCV to maintain expression of the miRNAs.

We used stringent criteria to conclude that MCV-mir-M1 is a bona fide miRNA. The miRNA we identified is detectable via Northern blot analysis (Figure 3.2) and is active at directing the inhibition of reporter gene expression, most likely through a RISC-mediated mechanism (Figure 3.3C). Furthermore, fine mapping studies of the MCV-mir-M1 and its cognate * 3P sequence (the strand that is less efficiently incorporated into RISC) results in a predicted duplex secondary structure with 2-3 nucleotide 3' overhangs- a hallmark of both microprocessor and Dicer processing. Combined, these results prove MCV-mir-M1 is a bona fide miRNA. A notable caveat to our approach is the possibility that we have missed other possible *bona fide* miRNAs encoded by MCV. Indeed, Vmir predicted four candidates that scored higher than MR17 in the MCC350 genome (Figure 3.1A). While Vmir is a useful starting point for identifying novel miRNAs in viral genomes, a high Vmir score alone is relatively uninformative due to the possibility of false positives. Therefore, ruling out the existence of additional MCV miRNAs is not possible until exhaustive, saturated small RNA cloning is performed on RNA obtained from all stages of the virus lifecycle (e.g. persistent and lytic infection). Obviously, these studies await the development of laboratory models of MCV infection.

What is the function of the MCV miRNA? Two lines of reasoning strongly suggest that MCV-mir-M1 functions to autoregulate early viral gene expression at late

times post infection. First, every polyomavirus we have examined in detail, including the SV40-like polyomaviruses (SV40, JCV and BKV) and MuPyV encode miRNAs at late times of infection that direct the cleavage of early RNA acting to autoregulate early protein levels at late times during infection (Seo et al., 2008; Sullivan et al., 2006; Sullivan et al., 2005; Sullivan et al., 2008). The genomic location, antisense and complementary to the early RNAs, supports a similar activity for MCV-mir-M1. Second, we have demonstrated that a chimeric reporter containing a portion of the early RNAs is specifically downregulated by expression of MCV-mir-M1. Importantly, these results do not rule out a potential additional function for MCV-mir-M1 in regulating a cellular target. However, since MCV-mir-M1 shares no sequence identity with any of the other known Polyomaviral miRNAs, it seems highly unlikely that these miRNAs could bind to and regulate the same cellular target. Thus, our results strongly suggest that a major function of the MCV miRNA, similar to the other Polyomaviral miRNAs, will be to autoregulate early gene expression at late times of infection.

Finally, the fact that the MCV miRNA is likely only expressed during lytic infection (when the late genes are expressed), limits the potential utility of it as an anti-viral drug target. To date, MCV is associated with tumorigenesis in cases where the viral genome has integrated into the host genome in a stable fashion and is likely only competent to express early gene products. It is currently unclear if any of these tumors are undergoing active lytic infection. Nonetheless, blocking the MCV miRNA function will do little to inhibit the tumorigenic activity of T antigens that are expressed from the tumor genome.

However, as the MCV field is brand new, it remains possible that lytic infection may indeed one day be linked to pathogenesis, and in that case, MCV-mir-M1 is a plausible therapeutic target. Irrespective, our study provides a proof of principle that viral miRNAs can rapidly be identified in newly emerging pathogens.

3.4 MATERIALS ANS METHODS

3.4.1 Computational prediction of MCV miRNA pre-cursors and miRNA northern blot analysis.

Vmir (Grundhoff, Sullivan, and Ganem, 2006; Sullivan and Grundhoff, 2007; Sullivan et al., 2005) was used to predict candidate pre-miRNAs in the MCV genome (The accession numbers are; MCC350: EU_375803, MCC339:EU_375804). The candidates were cloned into pcDNA3.1 puro, transiently transfected into human embryonic kidney cells (HEK) 293T. Total RNA was harvested using an in house Trizol-like reagent (2M Guanidinium Thiocyanate, 20mM Citrate buffer (PH 4.5), 5Mm EDTA, 0.25% Sarkosy, 48% saturated phenol (PH 4.5), 2.1% isoamyl alchol, 0.5% beta-mercaptoethanol, 0.1% 8-Hydroxyquioline, 0.0025% Coomassie blue) and Northern blot analysis was conducted as previously described (Seo et al., 2008) using the following probes:

MCV5' probe: GGAACCAGTGTACCTAGAAATTCTTCCAGAACGTA

MCV 3' pobe: AGACCACCAATTCAGGAAGAGAATCCAGCACACCCA

MCV loop: CACACCCAATGGAACCAGTGTAC

3.4.2 Small RNA library generations and fine mapping of MCV miRNA.

For fine mapping of the MCV miRNA, a library was generated as previously described (Seo et al., 2008). Briefly, small RNA (10 -40 nucleotides) was harvested from 293T cells transfected with the MCC350 miRNA expression plasmid. The small RNA was ligated and TA cloned into PCR2.1. miRNA specific primers were used in a PCR reaction to map both the 3' and 5' ends of the MCV miRNAs. The primers used were:

5' linker forward primer: GTTGATCAGAGCCCAGGG;

3' linker backward primer: ATTGATGGTGCCTACAG;

MCV 5p forward primer: AGTGTACCTAGAAATTCC

MCV 5p backward primer: GTTCTGGAAGAATTTCTA

MCV 3p forward primer: ATTCAGGAAGAGAATCCA

MCV3p backward primer: GTGTGCTGGATTCTCTTC

3.4.3 DNA constructs, transfection and Luciferase assays.

All DNA constructs were confirmed by sequence analysis. pCDNA3.1MCVmiRNA (MCC350), that expresses the MCV miRNAs, was generated by subcloning into the Hind III/Xba I sites of pCDNA3.1puro expression vector (Sullivan and Ganem, 2005). A plasmid containing the entire MCV genome (kindly provided by Drs. Chang and Moore, University of Pittsburgh) served as template to subclone the region of the MCV genome containing the MCV pre-miRNA as well as ~1Kb of flanking regions. The resulting plasmid was named "pcDNA3.1puroMCV350miRNA". To construct the MCV339 miRNA expression vector , we conducted PCR mutagenesis using

pcDNA3.1puroMCV350miRNA as the template. As a negative control, we engineered a deleted version of pcDNA3.1puroMCV350miRNA in which 300 nucleotides of sequence, encompassing the pre-miRNA and surrounding regions (nts 1017-1318 of the MCC350) was deleted. The luciferase reporter assays were performed as previously described (Seo et al., 2008) in HEK 293 cells. The reporter contains a single copy of a 300 nucleotide sequence (1051-1350 nt positions from one of the MCC genomes MCC350**)** corresponding to the complementary and flanking regions of the early transcripts. As a transfection control, Renilla luciferase levels were normalized to a co-transfected firefly luciferase reporter as previously described (Seo et al., 2008).

Chapter 4: Inactivation of RNAi promotes the antiviral response in mammalian cells

4.1 Introduction

RNA interference is an evolutionarily conserved process to direct post-transcriptional regulation of gene expression by small RNAs. These small RNAs can enter a protein complex (RISC) that binds directly to mRNA targets resulting in specific inhibition of translation. Small RNAs and RNAi are important modulators during virus-host interactions (Umbach and Cullen, 2009; Sullivan, 2008). While the role of RNAi as an antiviral defense is well established in plants and invertebrates, it is still a hotly-debated issue in mammalian systems (Umbach and Cullen, 2009; Van Rjj et al., 2006; Ding and Voinnet, 2007). Artificial transfection with small interfering RNAs (siRNAs) complementary to viral genome efficiently limits the viral replication and protects host from lytic viral infection. However, it is not enough to prove that the RNAi is a natural intrinsic antiviral response. Some reports show that mammalian viral proteins can inhibit RNAi activity (Haasnoot et al., 2007; Lichner et al., 2003). However, these studies were not conducted in the context of infection and only involved exogenous over-expression of the proteins. Another study shows that knocking-out a RNAi machinery component in mammalian cells increases the replication of a specific virus because of the loss of endogenous cellular miRNA, which is fortuitously complementary to viral transcripts (Otsuka et al., 2007). To date, no viral specific siRNAs have been detected in studies

conducted by small RNA cloning or in deep sequencing of mammalian cells while small siRNAs associated with the antiviral response are readily detectable in plants and insects during infection (Ding and Voinnet, 2007).

DNA viruses make long double stranded RNA by overlapping transcription. RNA virus also produces double stranded RNAs during virus life cycle. RNA interference pathway elicits the production of virus-derived small interfering RNAs (viRNAs) and induces specific gene silencing by viRNA-RNAi effector complexes. miRNAs are another classes of small RNAs. The mature 22 nucleotide products are finally incorporated into the RNA–induced silencing (RISC) complex. The functions of miRNAs depend on the complementarities between miRNA and the target mRNA site (Kim et al, 2009). While miRNAs function to regulate gene expression for development and cell homeostatsis, it is not well characterized whether it controls endogenous innate immune response genes, specially interferon stimulated genes.

In mammalian cells, interferon production is a primary defense response to viral infection (Randall and Goodbourn, 2008). Interferon induction is initiated by cytoplasmic pattern recognition receptors (PRR). PRRs such as MDA5 and RIG I sense a viral double stranded RNA structure in a size and structure dependent manner (O' Neil and Bowie, 2009), typically as an intermediate during viral replication. Such recognition transducers signal through the common molecule MAVS/IPS-1 and amplify the signal through NF-kappaB or IRF-3 leading to interferon production. Double stranded RNAs are also recognized by PKR. Activated PKR phosphorylates eIF2 alpha, causing global translational inhibition. Lastly, RNAase L pathways also activate a MAVS-signaling

pathway, increasing endogenous double-stranded RNAs, which are not well characterized (Malathi K, 2007).

It has recently been shown how miRNA's activity is regulated in cytoplasm in cellular stress. Leung et al.,(2011) discovered pADPr has new post-transcriptional regulatory function by inhibiting miRNA activity in cytoplasm. Macromolecule, poly(ADP-ribose) pADPr, has been reported to play an important role in DNA damage repair, chromatin remodeling, and transcription in nucleus of eukaryotic cells. However, cytoplasmic PARP complex aggregate each other by poly ADP-ribosylation modification under cellular stress. Interestingly, cytoplasmic proteins in stress granule are poly ADP ribosylated in response to cellular stress and the modification of Argonaute protein results in the decrease of global miRNA activity. Even though the biological function of cytosolic PARPs remains to be investigated, PARP12 is one of interferon stimulated genes (ISGs), it has an ADP-ribosyltransferase activity. Recently it reveals that the long isoform of PARP12 has an antiviral activity for the broad range of alphaviruses and RNA viruses. PARP 13 was originally characterized as ZAP, a host factor restricting the retrovirus infection. It is speculated that PARP13, defective of PARP activity, poly ADP-ribosylated by other cytosolic PARP enzyme and results in the inhibition of miRNA silencing. It recently has been shown that PARP13.2, shorter isoform among two isoforms of PARP13, binds to RIG-I in response to viral infection and stimulates type I interferon and inflammatory cytokines. It also shows that long form of PARP13 (PARP13.1) has a stronger antiviral activity by PARP like domain.

For this study, I established cell based reporter assay to monitor RNAi activity in 293 cells to determine whether RNAi plays an antiviral role in mammalian cells. Contrary to what would be predicted if RNAi is an antiviral response in mammalian cells, it shows that RNAi is strongly inhibited at early time points after viral infection. Studies with a chemical mimic of viral infection (poly I:C) implicate that the innate cellular immune response is responsible for this inhibition. This study describes a previously unknown mechanism that the depression of RNAi is caused by the host innate immune response and poly ADP ribosylation in mammalian cells.

4.2 Result

4.2.1 Triggering of the antiviral response blocks RNAi.

It is unclear how RNAi activity is modulated in response to viral infection in mammalian cells. There is no convincing data showing that RNAi plays a crucial role in host defense in response to viral infection in mammalian cells. I generated reporter cell lines to monitor RNAi activity (Figure 4.1) to test the idea that RNAi may only be active against viral infection in mammalian cells under conditions in which the host cell is undergoing an anti-viral response. (Note: the antiviral response changes numerous signaling pathways and dramatically alters cellular physiology). I generated stable cell lines expressing destabilized renilla luciferase reporters containing 4 copies of viral miRNA binding sites (Figure 4.1A). Stable cell lines that also express an exogenous miRNA that targets the reporter," reporter cell line" show that the renilla luciferase activity was reduced, and the mRNA level of renilla luciferase was less

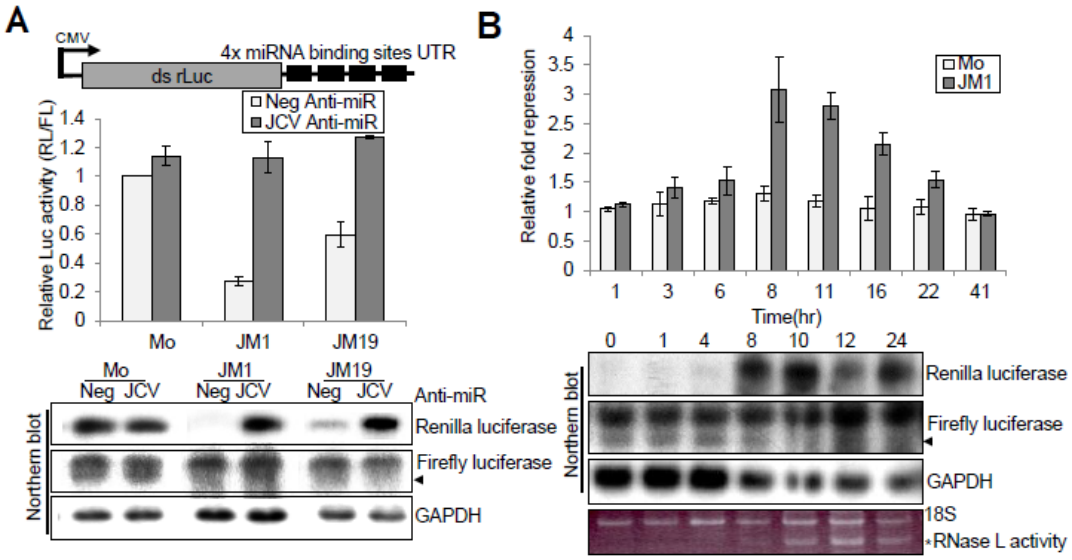

Figure 4.1 A trigger of antiviral response inhibits RNA interference (RNAi).
(A)Mother cell line (Mo) expresses both a Renilla Luciferase (Ren Luc) and a Firefly Luciferase (FF Luc) construct. JM1 and JM19 cells are derived from Mo cells and express an exogenous JCV viral miRNA that directs RNAi repression against Ren Luc. Transfection of an Anti-miR de-represses RNAi (grey bars transfected with specific JCV Anti-miR). Northern blot analysis shows depression of RNAi occurs at the level of RNA.
(B) Dual luciferase activity assay using parental cell (Mo) or microRNA reporter cell (JM1). Mo or JM1 cells were transfected with 0.5µg pI:C, a potent activator of the antiviral response. Luciferase activity was measured at indicated time. The depression of the RNAi reporter is only observed in JM1 cells. To obtain the relative fold repression, the luciferase activity (RL/FL) in pI:C treated cells was normalized to the luciferase activity (RL/FL) of untreated cells. Northern blot analysis of JM1 cells treated with pI:C. Note the cleaved 18S rRNA, indicative of RNaseL activity observed starting at about 6 hours post-pI:C

expressed compared to control cell lines which only contain reporters without miRNA expression (Figure 4.1A). To confirm that the reduced RNAi activity is due to miRNA activity, I transfected the reporter cell lines with 2'-O- methylated oligonucleotides (control and viral miRNA specific Anti-miR). The antisense inhibitor increased the luciferase activity and renilla luciferase mRNA level of reporter cell lines. Additionally, I transfected poly I:C (a viral dsRNA mimic), to trigger the antiviral response in the absence of viral gene expression (Figure 4.1B). All treatments described resulted in inhibition of RNAi . These surprising results oppose our original hypothesis and suggest that mammalian cells turn off the RNAi response upon sensing viral infection. These findings have important implications as to the contentious debate of whether or not RNAi can serve as an antiviral process in mammalian cells.

4.2.2 Antiviral-mediated De-repression of RNAi occurs post-dicer

To consider what component of the RNAi machinery is affected by the antiviral response, I determined the steady state level of miRNA in reporter cell lines (Figure 4.2A). I performed miRNA Northern blot analysis on RNA harvested from the RNAi reporter cells under normal conditions and from cells treated with poly I:C. I probed for the two viral miRNAs as two exogenous miRNAs target reporters. The steady level of miRNA was equal in both poly I:C treated and untreated cells. Furthermore, endogenous cellular miRNAs (hsa-LET-7) were detected in identical levels in both cells. This demonstrates

4.2.3 RISC mediated cleavage inhibited by poly I:C

As RISC functions downstream of Dicer, I performed an experiment to determine if RISC activity is inhibited by antiviral signaling. I adapted the *in vitro* Ago2 cleavage assay used in Zebrafish system to mammalian cells. I applied *in vitro* RISC assay using purified pre-mir 451 (Dharmacon), which has a process of Ago2 specific cleavage bypassing dicer step in the miRNA biogenesis (Cheloufi et al., 2010). Treatment of Ago2 substrate RNA with mammalian cell lysate results in specific cleavage. Immunodepletion assay of Ago2 confirms the specificity of this assay. Lysate prepared from cells transfected with poly IC is defective in RISC – mediated cleavage demonstrating that RISC is blocked by antiviral signaling (Figure 4.2B).

4.2.4 Triggering the anti-viral response ADP-ribosylates Ago2

As RISC activity is changed by antiviral response, viral stresses will change the modification of the Ago2. Such modification will affect the RISC activity. Recently, Leung and colleagues (2011) demonstrated that treatment of cells with oxidative stress and inhibition of translation by drug induced pADP-R of human Ago1-4 which relieves miRNA-mediated repression. Immunoprecipititiaon of Ago2 followed by immunoblot with antibody that detects pADP-R modified proteins of Ago2 in the pI:C treated samples. Performing RNAi activity assay in the presence of a chemical inhibitor of PARP [3-aminobenzamide] results in a diminished relief of RNAi that is triggered by

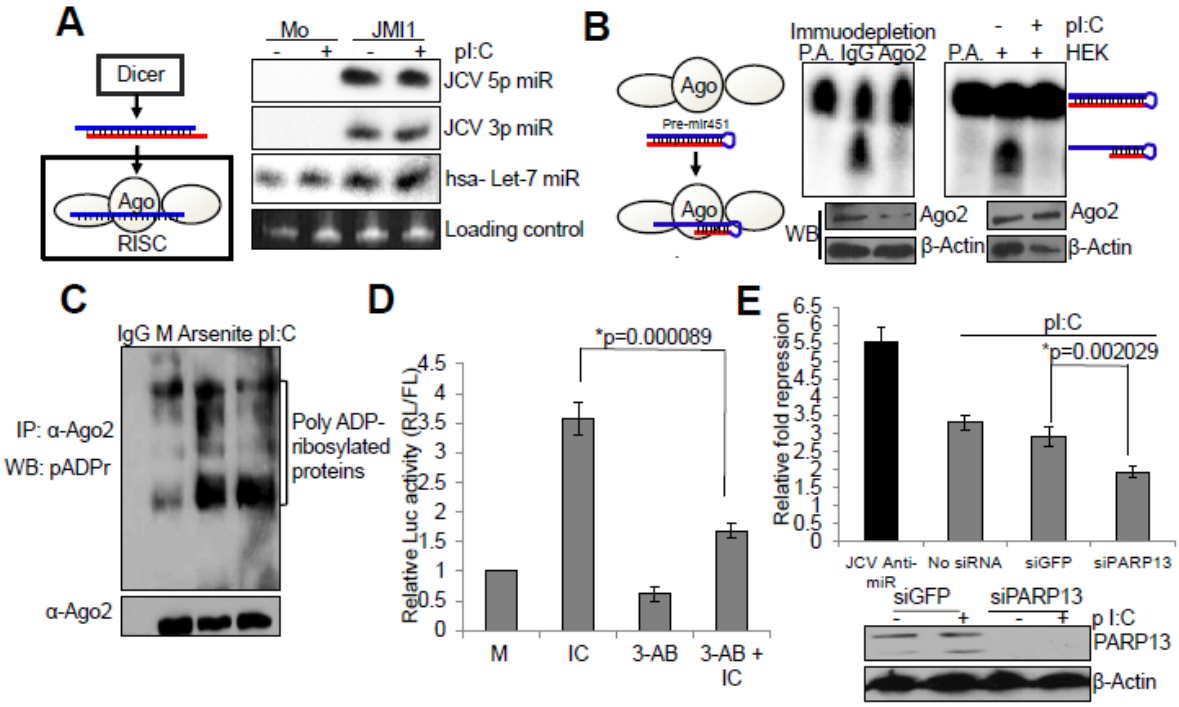
A
Dicer
Ago
RISC
Mo
JMI1
- + - +
pI:C
JCV 5p miR
JCV 3p miR
hsa- Let-7 miR
Loading control
B
Ago
Pre-mir451
Ago
Immuodepletion
P.A. IgG Ago2
P.A. + + HEK
- + pI:C
WB
Ago2
β-Actin
Ago2
β-Actin
C
IgG M Arsenite pI:C
IP: α-Ago2
WB: pADPr
Poly ADP-ribosylated proteins
α-Ago2
D
*p=0.000089
Relative Luc activity (RL/FL)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
M
IC
3-AB
3-AB + IC
E
pI:C
*p=0.002029
Relative fold repression
6.5
5.5
4.5
3.5
3
2.5
1.5
1
0.5
0
JCV Anti-miR
No siRNA
siGFP
siPARP13
siGFP
siPARP13
- + - +
p I:C
PARP13
β-Actin

Figure 4.2 A trigger of antiviral response inhibits *in vitro* RISC mediated cleavage and RNAi through poly ADPribosylation.

(A) The antiviral response inhibits RNAi at the level of RISC. Northern blots analysis shows miRNA levels are unaffected by pI:C treatment. Equal amount of a viral miRNA that directs RNAi against Ren Luc from pI:C treated and untreated cells was detected and a endogenous cellular miRNA (hsa let-7 miR) also had similar levels in pI:C treated and untreated cells.

(B) pI:C inhibits RISC mediated cleavage. To validate the Ago2 specific cleavage of substrate in an *in vitro* RISC mediated cleavage assay, Ago2 was immunodepleted with a Ago2 polyclonal antibody (7C3) or IgG and supernatant was added into the assay. Crude extracts from pI:C treated or untreated cells were used in the *in vitro* RISC mediated cleavage assay using 5' end radiolabled substrate (gel purified pre-miR 451). The cleavage product of pre-miR451 was not shown in Ago2 immunodepleted or pI:C treated sample. The cytosolic protein amount used in *in vitro* RISC assay was representatively measured by Ago2 , β-Actin.

(C) Antiviral triggers result in the poly ADP-ribosylation of Ago2. HEK 293 cells were treated with pI:C. Immunoprecipitated Ago2 from whole cell lysates were analysed by western blot with an antibody specifically detecting poly(ADP)-ribose. A 90min treatment with 250μM Sodium arsenite served as a poistive control of Ago2 poly ADP-ribosylation (Leung ak et al, 2011). Equal amount of Ago2 IP was measured by Ago2 antibody.

(D) Treatment with 3-aminobenzamide (a PARP inhibitor) blocks pI:C-mediated inhibition of RNAi. JM1 reporter cell lines were transfected with 0.5 μg pI:C. RNAi activity was measured after treatment with 20 mM 3-aminobenzamide.

(E) The knockdown of PARP13 mitigates the inhibition of miRNA activity after treatment with pI:C. JM1 reporter cells were transfected with an siRNA against eGFP or PARP13. Relative fold repression was measured as the activity of luciferase (RL/FL) in the pI:C treated cells normalized to untreated cells. The relative fold repression by JCV Anti-miR was shown as maximized fold repression. The experiment independently repeated more than three times. This is a representative graph of data from independently repeated trials.

that the miRNA maturation was not changed in cells undergoing the antiviral response. Therefore, it suggests that RISC, downstream of Dicer, must be regulated to inhibit RNAi activity in response to viral infection. Triggers of the antiviral response, specifically poly I:C, activate PKR and lead to nonspecific translational inhibition by phosphorylating eIF-2 alpha (Randall and Goodbourn 2008). I determined whether triggers of the antiviral response alter the amounts of RISC components. Ago2 proteins are major protein in RISC complex and Dicer protein, RNase III protein, which affect to RNA silencing efficiency. I measured steady level of Ago2 and Dicer protein by western blot. The amount of protein was not changed at the time point shown in the inhibition of RNAi activity (data not shown). Even though I cannot exclude the possibility the change of other protein of RISC complex having a short half- life, I concluded that major RNAi proteins, consistent with previous miRNA biogenesis data, are not changed by nonspecific translational inhibition. The alteration of RNAi activity likely occurs in RISC or RISC loading, as the steady state level of miRNA does not change after triggering the antiviral response. Therefore, I investigated what molecular event of RISC occurs in cells undergoing the antiviral response.

transfection of poly IC. Leung et al., demonstrated that the cytosolic PARP13 is a key components of stress mediated relief of RISC activity. Using siRNA knockdown of PARP13, I observed decreased poly IC-triggered repression of RNAi. In summary, these results argue that triggering the mammalian antiviral response results in the inhibition of RISC activity that is partially dependents on PARP13 activity and poly ADP-ribosylation of Ago2.

4.2.5 Viral infection inhibits RNA interference

To test if triggering of antiviral responses with actual virus infection changes the RNAi activity, I infected reporter cell lines with three different viruses, Herpes Simplex 1 (a DNA virus), Sendai virus, and Flu wild type and NS mt S42D (both negative strand RNA viruses known to elicit a strong antiviral response) (Figure 4.3A). Infection with HSV-1 and Sendai virus results in relief of RISC activity. Infection with Flu A induces only minimal relief of RISC. However, infection with Flu mt relieves RISC repression, demonstrating that this effect is due to the virus activating the host antiviral signaling response as opposed to the virus directly blocking RISC. *In vitro* RISC assays confirm that infection with HSV-1 inhibits Ago2-mediated cleavage (Figure 4.3B). Furthermore, Ago2 displays increased ADP-R in response to infection with HSV-1, Sendai virus or Flu mt. Immunoprecipitation of PARP13 studies show that PARP13 is itself ADP-ribosylated in response to infection with Flu mt or HSV-1(Figure 4.3C). Furthermore, PARP 13 is shown in greater association with Ago2 upon infection (Figure 4.3D). These findings connect PARP13 activity and association with Ago2 to viral infection (Figure 4.3E). To determine if ADP-R is required for viral-mediated relief of RISC activity, we treated our RNAi reporter cells with 3AB (3-Aminobenzamide) during infection with HSV-1. Treatment with 3AB suppresses the RNAi relief observed with HSV-1 infection, and this relief correlates in degree with the amounts of ADP-R detected on Ago2 (Figure 4.3F). Therefore, mammalian cells respond to infection with diverse viruses by ADP-ribosylating PARP13 and Ago2, thereby inhibiting RISC activity.

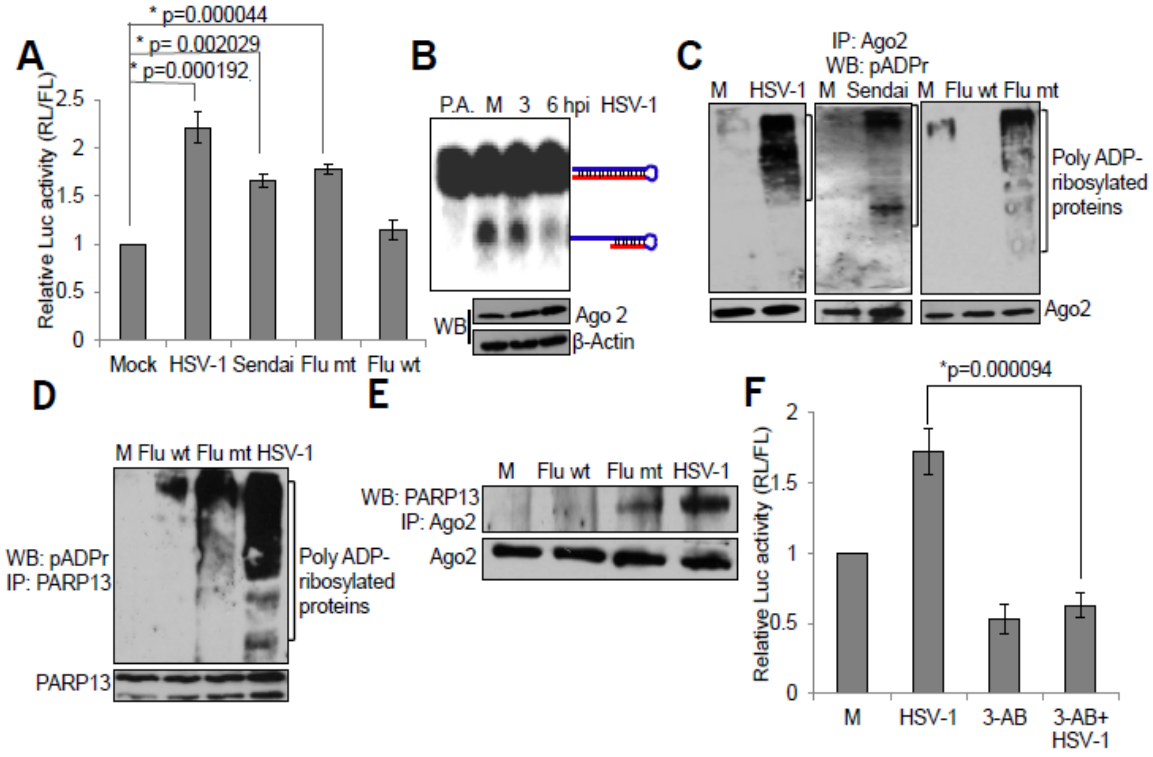
A
* p=0.000044
* p= 0.002029
* p=0.000192
Relative Luc activity (RL/FL)
2.5
2
1.5
1
0.5
0
Mock
HSV-1
Sendai
Flu mt
Flu wt
B
P.A. M 3 6 hpi HSV-1
WB
Ago 2
β-Actin
C
IP: Ago2
WB: pADPr
M HSV-1 M Sendai M Flu wt Flu mt
Poly ADP-ribosylated proteins
Ago2
D
M Flu wt Flu mt HSV-1
WB: pADPr
IP: PARP13
Poly ADP-ribosylated proteins
PARP13
E
M Flu wt Flu mt HSV-1
WB: PARP13
IP: Ago2
Ago2
F
*p=0.000094
Relative Luc activity (RL/FL)
2
1.5
1
0.5
0
M
HSV-1
3-AB
3-AB+ HSV-1

Figure 4.3 Viral infection inhibits RNA interference.

(A) JM1 reporter cell lines was infected with HSV-1, sendai, or Influenza A. Cells were assayed for RNAi activity: HSV-1 strain 7 (a DNA virus) at 10 hours post infection (hpi). Same cell line was infected with negative strand RNA viruses, sendai virus and influenza A virus. It was infected with 50 HA/ml sendai virus (indiana strain) at 6 hpi. It was also infected with 2 MOI influenza A virus Ud72 wild type, or Ud72 mt(S42D) into JM1 cell line at 12 hpi.

(B) HSV-1 infection inhibits RISC mediated cleavage. *in vitro* RISC mediated cleavage assay was performed as shown in Fig.2B. 293 cell extracts infected with HSV-1 were incubated with the P^{32} radiolabed pre miR-451 to observe *in vitro* RISC mediated cleavage activity. The cytosolic protein amount used was representatively measured by Ago2, β-Actin.

(C) Viral infection results in the poly ADP-ribosylation of Ago2. For the detection of poly ADP-ribosylatin of Ago2, the experiments were conducted as shown by Fig.2C. 293 cells were infected with HSV-1, Sendai virus, Flu A wild type or Flu A mt. Immunoprecipitated Ago2 from whole cell lysates was analyzed by western blot with poly(ADP)-ribose antibody. Immunoprecipiated Ago2 was measured with Ago2 antibody.

(D) Viral infection induces the poly ADP-ribosylation of endogenous PARP13. PARP13, a member of the PARP family, is localized in cytoplasm and plays a crucial role in the inhibition of RNAi during antiviral response. Immunoprecipitated PARP13 from whole cell lysates was probed with pADPr antibody. The amount of immunoprecipitated PARP13 was measured with a PARP13 antibody.

(E) Viral infection increases the interaction between PARP13 and Ago2 in the cytoplasm. 293 cells were infected with HSV-1, Flu wild type and Flu mt, respectively. Whole-cell extracts were subjected to Ago2 immunoprecipitation followed by immunoblotting for the indicated protein.

(F) The inhibition of PARP activity blocks the depression of RNAi after infection with HSV-1. HSV-1 triggered RNAi inhibition was measured after treatment with 20mM 3-aminobenzamide.

4.2.6 The components of the innate response are responsible for the inhibition of RNAi.

To investigate what components of the antiviral response are important for relief of RISC-mediated repression, I assayed a possible role for interferon. This assay shows that treatment with interferon is not sufficient to relieve repression of RISC, but rather augments the relief of RNAi activity that is observed with pI:C treatment. Therefore, detection of virus infection via an intracellular PRR is required to alleviate RISC activity. The previous evidence shows that an intracellular innate immunity pathway triggered by dsRNA is responsible for the inhibition of RISC. The following two points support this assumption. 1) Viruses produce double-stranded RNAs during virus replication. 2) Transfection with a mimic of double stranded RNAs are sufficient to trigger the antiviral response and inhibit RNAi. There are three well-defined intracellular dsRNA pathways, 1) MAVS 2) RNase L, 3) PKR. I performed the poly ADP-ribosylation of Ago2 assay using mouse embryonic fibroblast cells (MEFs), which are null for either MAVS, RNase L, or PKR . MEFs are null of MAVS or RNase L both show reduced ADP-R of Ago2 after infection with HSV-1, whereas PKR knockout MEFs shows no difference in the poly ADP-ribosylation of Ago2 (Figure 4.4A). Next, I focused on MAVS protein as a likely PRR required for the inhibition of RISC activity. Indeed, siRNA-mediated knockdown of MAVS leads to decreased poly IC-induced inhibition of RISC activity. I confirmed with Western blotting to assess siRNA mediated knock down of the target protein (Figure 4.4B). These data show that MSVS contributes to the repression of RISC that is triggered by viral infection or chemical triggers of the antiviral response.

4.2.7 miR-17 regulates interferon stimulated genes

The question arises as to what benefit mammalian cells receive from inhibiting RISC during viral infection. ADP-R of Ago leads to depression of both siRNA-mediated cleavage and miRNA-mediated repression (Leung et al., 2011 and data not shown). We hypothesized that relief of miRNA-mediated regulation could allow for enhanced expression of ISGs, many of which are toxic to cells and therefore likely to be under multiple layers of regulation. We first utilized two different bioinformatic approaches on literature-annotated ISGs. Additionally, ISG 3' UTRs are also predicted to have a higher density of evolutionarily-conserved miRNA binding sites (Figure. 4.5A). Combined, these analyses support that ISGs, especially those associated with cell death, are particularly susceptible to miRNA-mediated regulation. We also undertook an independent line of experimentation utilizing DLDExp5-/-, a human cell line hypomorphic for miRNA expression due to an engineered mutant Dicer. We treated miRNA hypomorphic cells or cells derived from the isogenic parental line that expresses readily-detectable miRNAs with IFN and performed microarray analysis to look for differences in induction of ISGs . Indeed, the hypomorphs express numerous IFN-induced transcripts at higher levels than the parental cells (Figure 4.5B). Using the Sylamer algorithm, we analyzed the differentially expressed transcripts for evidence of miRNA regulation. one miRNA family, miR-17/93 dominates (Figure 4.5B). Interestingly, this family of miRNAs also was amongst our top-ranked miRNAs bioinformatically-predicted to regulate ISGs and has been previously shown to have a

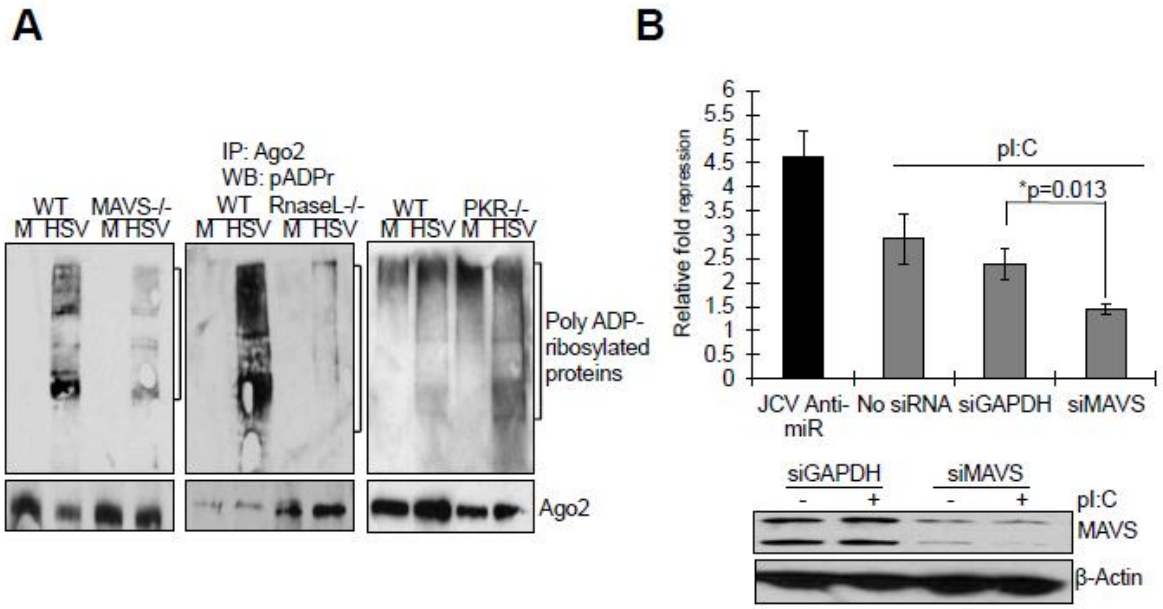

Figure 4. 4 The antiviral response pathway is involved in the inhibition of RNAi.

(A) MAVS and RNase L are involved in the virus induced poly ADP-ribosylation of Ago2. The detection of poly ADP-ribosylated Ago2 in MEFs was performed. Immunoprecipitated Ago2 from whole cell lysates was analyzed by western blot with poly(ADP)-ribose antibody. Immunoprecipitated Ago2 amount was measured with an Ago2 antibody.

(B) MAVS is required for maximal depression of RNAi by treatment with pI:C. JM1 reporter cells were transfected with siRNA against GAPDH or siRNA against PARP13. Relative fold repression was measured as the activity of luciferase (RL/FL) in the pI:C treated cells normalized to untreated cells. The relative fold repression by JCV Anti-miR was shown as maximized fold repression and a positive control in this assay. Knockdown of MAVS was detected with western blot in siMAVS treated cells. The experiment independently repeated more than three times and a representative graph is presented.

proviral effect via an unknown mechanism (Santhakumar et al., 2010). To test if miR-17/93 conveys a proviral effect due to it suppressive effects on ISGs, we transfected cells with a miR-93 or miR-17 mimic and infected them with HSV-1 in the presence or absence of IFN treatment for 3 hours. Pre-expression of exogenous miR17/93 results in a >3-fold increase in HSV virus yield in the presence of IFN but only a 2-fold increase without IFN. This extends our understanding of how miR-17/93 is pro-viral by implicating a role for these miRNAs in limiting the effects of IFN (Figure 4.5C). Next, we directly examined whether triggering of antiviral response relieves ISG repression. To confirm that a predicted ISG is regulated by mir17/93. I transfected whole cloned IRF9 UTR reporter. It was repressed with 1.8 fold. Repression by mi17/93 is decreased into 1.2 fold indicating depression under antiviral response triggered conditions (Figure 4.5D). Thus, these data show that relief of miRNA repression mediated by antiviral response results in release of ISG transcripts from RISC repression. As miRNAs are endogenous

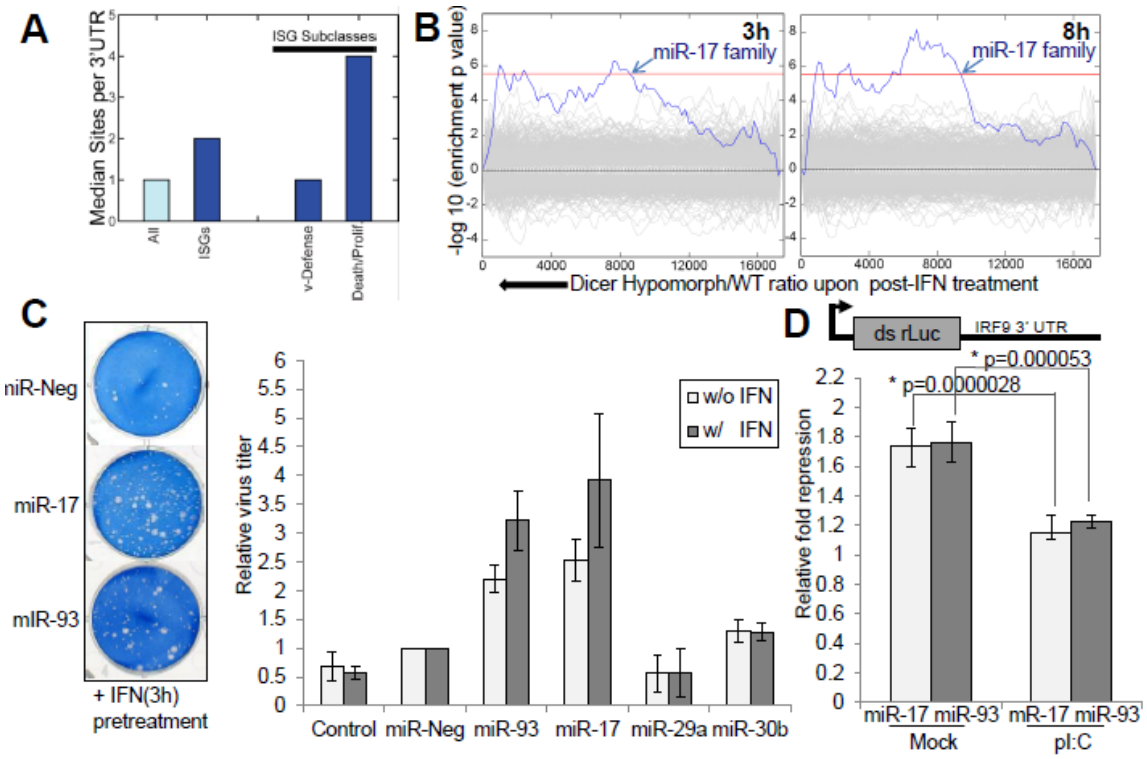

Figure 4.5 miR-17 family regulates interferon stimulated genes and promotes HSV-1 infectivity.

(A) Bioinformatics strongly predict interferon responsive genes are under miRNA-mediated Regulation. (Data from Rodney Kincaid)

(B) Mir17 regulates interferon stimulated genes. (Data from Rodney Kincaid)

(C) Addition of mir17 promotes HSV-1 infectivity. DLD1 Dicer hypomorph cells were transfected with the respective miRNA mimic. After 24 hrs, cells were treated with 1000 U/ml interferon for 3 hours and then infected with 1 MOI HSV1. Virus was collected after 24 hours and quantified by a plaque assay to observe the infectivity of HSV-1. (Data from Phanaksri Teva)

(D) An interferon stimulated gene, IRF9, are repressed by miR-17/miR-93 and after treatment with pI:C is derepresssed. miRNA-directed repression was tested with a renilla luciferase reporter with a whole IRF9 UTR predicted to be targeted by miR-17 family. Renilla luciferase and Firefly luciferase reporters were co-transfected into 293T cells with 5 nM control miRNA, miR-17 or miR-93 mimics for 24hrs. To observe the de-repression of miRNA directed repression, 0.5ug pI:C was transfected 24 hours after transfection with reporter plasmids. To obtain the repression fold, the luciferase activity (RL/FL) in miR-17 or miR-93 was normalized by the luciferase acitivity (RL/FL) of control miRNA treated cells in the pI:C treated or untreated cells.

products of the RNAi machinery, these results also show that some RNAi products can be considered proviral under IFN-rich conditions.

4.3 Discussion

RNAi serves to actively defend against viral infection in invertebrate organisms (Ding and Voinnet., 2007). However, it has been debated whether RNAi plays an antiviral role in mammalian cells. It has been unclear how RNAi activity is modulated in response to viral infection in mammalian cells. One possible reason is that no one assayed RNAi activity under cellular conditions where an antiviral response was engaged. To test if RNAi serves as an antiviral response in mammalian cells, I have developed a sensitive method to assay the alteration of RNAi activity. However, the result unexpectedly leads to the inhibition of RNAi. As a mechanism, RNAi is inhibited by the poly ADP riboylation of Ago2 after viral infection and antiviral response through double stranded RNAs pathway are eventually linked to inactivation of RNAi. It has been reported that RNAi activity can be changed under cellular stress. It has relieved miR-122 mediated translational repression under cellular stress (Bhattacharyya et al., 2006). Vasudevan et al., (2007) shows that serum starvation stress upregulates miRNA mediated gene expression. It has been shown that the cellular stress inhibit miRNA activity through Ago2 relocalization or polyADPribosylation of Ago2 (Detzer et al., 2010; Leung et al., 2011). My study shows that cellular response in respond to viral infection modulates RNAi activity through a molecular pathway. Completion of this study detailed a previously unknown “cross talk” between RNAi and the host innate immune response in

mammalian cells (Figure 4.6). It is an unresolved question how interferon stimulated genes are promoted under condition of translationally global downregulation after viral infection. As one of possible explanation, miRNAs can be possible key. miRNAs mute the target gene under normal status without triggering of antiviral response, but once virus triggers antiviral response, miRNA targets are relieved. I showed miR-17 family represses interferon stimulated genes. However, it is required to clarify the identification of ISGs by other miRNAs. mir 142 and miR-548 are also highly scored to regulate the ISGs in bioinformatics analysis. The experimental demonstration will be a better understanding the regulation of ISG by miRNAs.

Signal transduction for RNAi depression is mediated by MAVS protein. However, the downstream area is open to question. Interestingly, According to proteomic analysis in Li et al.,(2011), the Ago2 is bound to TBK1 and TBK1 binding protein. Combined with my previous data, Ago2 can be regulated by MAVS downstream pathway. Also, as a my preliminary data, p38 inhibitor blocked RNAi inactivation by pI:C. According to paper, p38 pathways phosphorylate Ago2 protein and affect the localization to P-body or stress granule under the stress. P38 MAPK, a downstream of MAVS signaling, may regulate RNA interference after viral infection.

Accumulating data show miRNAs regulate immune response gene including interferon stimulated genes and RNAi is globally downregulated in cancer. Therefore, the small molecule discovery modulating microRNA activity eventually might play a major role to have a potential an intervention of viral infection.

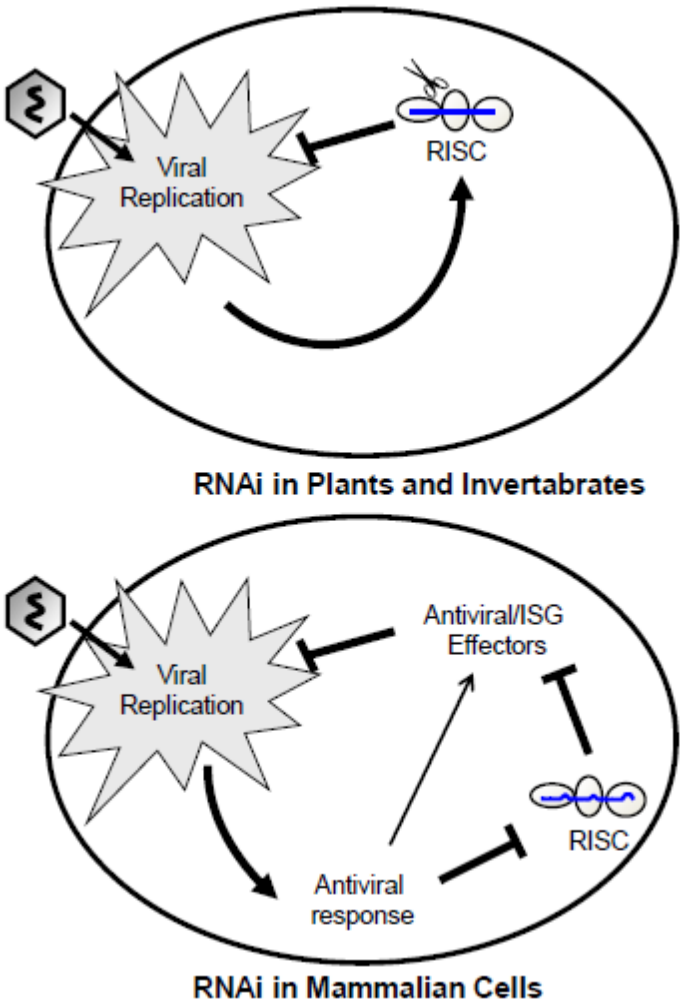

Figure 4.6 Model for Role of RNAi During Viral Infection
While RNAi directly blocks viral replication in plants and invertebrates, RNAi in some mammalian cells normally represses ISG/antiviral effectors via miRNAs, and therefore is inhibited at early times during the antiviral response to allow maximal expression of ISGs.

4.4 Materials and Methods

4.4.1 Plasmid Constructs

To perform the luciferase reporter assay for IRF9, the full length 3'UTR of IRF9 including the potential miR-17 target site was made by PCR using human cDNAs as a template. Forward and reverse primers include XhoI, XbaI sites, respectively. Amplified PCR product was subcloned into pcDNA3.1 puroRenLuc expression vector. pcDNA3.1puroRen Luc MCV miRNA binding site and pcDNA3.1puroFirefly Luc plasmids (Seo et al., 2009) were used to establish MM35 cell lines. MCV pri-miRNA including flanking region was subcloned from pcDNA3.1puroMCVmiRNA expression vector into pcDNA3.1hygro vectors using restriction enzymes, HindIII/XbaI. To explore miRNA-mediated derepression, perfectly matched JCV miRNA binding site were replaced with bulged JCV miRNA binding sites by PCR mutagenesis and cloning as shown by John et al.,(2003). The sequences are same with original vector except bulged region.

4.4.2 Stable cell line derivation

Stable Mo, JM1 cell lines have been described in McClure et al.(2011). MM35 cell lines were made by two antibiotic selections as shown in the derivation of JM1 cell lines. To make parental cell lines stably express both Rennila Luc and Firefly Luc reporter genes, 293 cell lines were puromycin selected co-transfection with pcDNA3.1puroRen Luc MCV miRNA binding site and pcDNA3.1puroFireflyLuc

plasmids. Stable clone were confirmed by Luciferase assay. Next, pcDNA3.1puroMCVmiRNA expression vector was transfected into parental cells and selected by hygromycin B. Stably cloned MM35 cell lines was used during experiments.

4.4.3 Cell culture and Reagents

HEK (Human embryonic kidney) 293 derived Mo, JM1, JM19, MM35 cells, wild type MEFs (Mouse embryonic fibroblasts) and MAVS knockout MEFs (gifted from Chen lab, UT southwestern medical school) were cultured with DMEM supplemented with 10% fetal bovine serum and antibiotics. Wild type MEFs, RNase L knock out MEFs, and PKR knockout MEFs were maintained with RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. DLD1 Dicer hypomorph cells were cultured with McCoy's medium supplemented with 10% fetal bovine serum and antibiotics. The following reagents were commercially obtained; pI:C (in vivogen), 3-Aminobenzamide and Arsenite (Sigma), PARP13 antibody (Abcam), Monoclonal Ago2 antibody (sigma), Monoclonal mouse Ago2 antibody (Wako), Actin antibody (santa cruz biotechnology), pADPr antibody (Amersham), MAVS (Bethyl laboratories). Ago2 7C3 antibodies were kindly gifted from Hobman lab (University of Alberta, Canada).

4.4.4 Dual luciferase reporter assay

Mo, JM1 cells were first seeded in a T75 flask at 80% confluency. The medium was removed and the cells were gently rinsed with 5 mL of D-PBS. One mL of trypsin-EDTA was added and the flask was tapped vigorously for approximately 30 seconds to

detach and resuspend cells. The trypsin reaction was quenched by resuspending cells in 9 mL of growth medium using a serological pipette. Cells were centrifuged at 3300 x g for 3 minutes and the supernatant was discarded. The pellet was resuspended in 10 mL of growth medium by gently pipetting. A single T75 flask contains enough cells to seed approximately three or four 12-well plates. For one 12-well plate, 3.3 ml of cell resuspension was added to 10 ml of growth medium and 1000 µl was added to each well.

Treatment with poly IC

One day after seeding cells, each well of 12-well culture plate was transfected with 2 µl of Lipofectamine 2000 and 0.5 µg pI:C in 200 µl of DMEM. After mixing with reagents, The mixture was incubated for 15 min at room temperature. Standard poly I:C treatment lasted 8hrs before assaying RNAi activity except for the time course assay in which poly I:C treatment lasted 0-24hrs.

Viruses infection

HSV-1 strain 17 was obtained from Craig McCormick (Dalhousie University). Sendai virus was purchased from Charles River. Influenza virus A wild type and Flu mt S42D was gifted from Robert Krug (University of Texas Austin). JM1 cell lines or MEFs were infected with 10 MOI HSV-1, 50 HA/ml sendai virus or 2 MOI Flu A WT or Flu A S42D viruses at 37 °C. For luciferase assays, cells infected with Sendai, HSV-1 and Flu viruses were lysed at 6, 10, 12 hpi, respectively. The growth medium was removed using an aspirator and 250 µL per well of room temperature passive lysis buffer was added to each well. Cells were incubated for 15 minutes at room temperature to ensure lysis. The dual luciferase assay was performed using a Luminoskan Ascent Luminometer (Thermo

Electronic Corporation, Milford, MA). 50 µL of LAR II was added to read firefly luciferase enzyme activity and recorded the results. 50 µL of Stop & Glo was added to read renilla luciferase enzyme activity. Normalization compared treated cells to an untreated control. The replicates were averaged.

4.4.5 Transient assay to detect miRNA-mediated depression

$2x10^5$ 293T cells were seeded in a 12 well culture plate. A plasmid mix containing 100 ng Firefly luciferase expression vector, 100ng renilla luciferase expression vector and 1.6 µg empty vector or 5 nM miRNA mimics (control miR, MCV miR, JCV miR, miR-17, and miR-93) was transfected into cells for 18 hrs. The 5nM concentration of miRNA mimics showed a maximum derepression. Cells were treated with 250 µM Sodium Arsenite for 90 min or pI:C was transfected for 8 hrs. Cell were carefully washed with D-PBS and lysed with 1 X Passive Lysis Buffer (Promega). Luciferase activity was measured on all the above conditions.

4.4.6 Northern blot and miRNA northern blot analysis

Northern blot for miRNA detection was done as described in Seo et al., (2008). Total RNA for northern blot analysis was isolated from cells by Trizol (GIBCO BRL) reagent. RNA was run in a 0.8% agarose gel containing 0.22 M formaldehyde at 90V for 2 hrs. The RNA was transferred to a nylon membrane and fixed by UV cross-linking. The filter was hybridized at 55 °C overnight with a radioactively labeled probe in ExpressHyb solution (Clontech), then rinsed twice with 2 × SSC, 0.05% SDS at room temperature

and finally with 0.1 × SSC, 0.1% SDS for 60 min at 50°C. The probe was stripped from the blot with 0.5% SDS at 90°C for 10 min. The filter was then re-equilibrated in the ExpressHyb solution and reprobed with a new sequence. The hybridization signals were quantitated by scanning the membrane with a PhosphoImager (Molecular Dynamics) or film was added to the blot.

4.4.7 RNA interference

Chemically synthesized 21-nucleotide siRNAs was purchased from Dharmacon, and IDT DNA. Cells were twice transfected with 20nM siRNAs within 96 hrs. First, cells were transfected with 20 nM siRNAs collected after 48 hrs. The collected cells split into 12 well culture plates for the second siRNA treatment.

4.4.8 Western blot

Cytoplasmic lysis buffer was used to extract proteins. Buffer consisted of; 50Mm HEPES, pH 7.4, 150 mM NaCl, 1mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1mM DTT, 1 mM ADP-HPD (EMD) and Complete EDTA free protease inhibitor cocktail (Roche). Cell extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis and immunbloted antibodies specific to each experiment: (β-Actin, PARP13, MAVS).

4.4.9 *In vitro* Ago2 cleavage assay

We adapted a Zebrafish *in vitro* Ago2 cleavage assay into mammalian cell culture (Cifuentes et al., 2010).

Treatment with pI:C and virus infection

HEK 293 cells were used for the assay. Mock or pI:C was transfected into 12 well plates. One day after seeding cells, each well was transfected with a mixture of Lipofectamine 2000 and pI:C. Each well contained 2 µl of Lipofectamine 2000 and 0.5 µg pI:C in 200 µl of DMEM. poly I:C treatment proceeded for 8hrs. Infections were carried out under the same cell culture conditions. 10 MOI HSV-1 infections were harvested after 0, 3, 6 hpi.

Preparation of cell extracts

Cell extracts were prepared as described (Gunter et al.,2004). 5 x 10^9 HEK 293 cells were collected by centrifugation and washed with D-PBS. The cell pellet was resuspended in five pellet volumes of cell lysis buffer including protease inhibitor (Roche) and incubated for 10 min on ice and collected again by centrifugation. The cell pellet was resuspended in 2 pellet volumes and sonicated for 3 miniute. The cell debris was removed from the cell lysate by centrifugation at 1000 g for 2 min and the supernatant was further centrifuged at 15000g to obtain the cytoplasmic extract. The concentration of KCl of the extract was raised to 100mM by addition of concentrated KCl. The extract was then adjusted to a glycerol content of 10% and immediately frozen in liquid nitrogen.

Immunodepletion assay

25 µl of bead slurry was put into 0.5ml tubes. It was washed with buffer three times and then 4µg of rabbit Ig G or 4µg of polyclonal anti-Ago2 (7C3) was added bringing the volume to 400 µl. Beads were incubated with each antibody at 4 °C for 1 hr on a rotator to allow the antibody to bind to the beads. The sample was centrifuged at 4 °C and washed with buffer. 150-200 µl of cell extracts was transferred to the precipitated beads and incubated at 4 °C for 1 hr on a rotator. Supernatants were followed by *in vitro* RISC mediated cleavage assay and western blot.

in vitro RISC mediated cleavage assay

Gel purified pre-miR451 was purchased from Dharmacon. The sequence is

The pre-miR451 was freshly phosphorylated prior to use by incubating in a 1µM PNK buffer containing 10 units T4 PNK(NEB) for 30min at 37 °C , followed by purification with illustra microspan G-25 columns (GE healthcare). 1 µM of 5' end labled hairpin was incubated in 10 µl volume containing 20 Mm HEPES-HCL, pH 7.0. 100 mM KCL. The cleavage reaction was performed for 15 min at 37 °C. The final reaction volume was 30 µl. The reaction was stopped by the addition of 5 x gel loading buffer (25% glycerol, 0.5% SDS, 50mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) and proteinase K, incubated for 30 min at room temperature, and then heat denaturated (95 °C, 1 min).The samples were loaded directly onto a 15% denaturing polyacrylamide

gel. Radioactive Gels were exposed to Phosphor imager (Molecular Dynamics), or exposed to film.

Immunoblot

Cell extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis and immunobloted with each antibody: Ago2, β-Actin.

4.4.10 Detection of the poly ADP–ribosylation Ago2

293 cells were transfected with pI:C into 12 well culture plates or infected with HSV-1, sendai virus, and influenza virus A. MEFs in a 150 cm^2 cultured plate were infected with HSV-1. Cells were collected by scraping the culture flasks and washed twice with D-PBS. Buffer C was applied to the cell pellet as previously described. 40 min before harvesting cells, 0.5 mg/ml latrunculin B was added. Cell lysate was centrifugated at maximum speed for 10 min, transferred into new tubes and treated with a final concentration of 10mg/ml cytochalasin B and 25 mM nocodazole. To detect the poly ADP-ribosylation of Ago2, 2ug of Ago2 monoclonal antibody (Sigma) or a mixture with mouse Ago2 and Ago2 monoclonal antibody (in MEFs, WAKO) was preincubated with protein-G beads. The beads were washed three times with buffer C for 15 min at 4 °C before applying sample buffer and heating for 10 min at 70 °C. Half of immunoprecipitated samples were used for the western blot. Immunoprecipitates were subjected to 10% SDS-polyacrylamide gel electrophoresis and immunobloted with each antibody: Ago2, PARP13, or pADPr.

4.4.11 Plaque-forming assay

miRNAs

Mission® miRNA mimics and negative control were purchased from sigma.

Cells and culture condition

DLD1 cells were cultured in McCoy's 5A medium (ATCC) supplemented with 100 U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, and 10% bovine serum albumin (BSA). Vero cells were cultured in DMEM (CellGlo) supplemented with antibiotics, L-glutamine and BSA. For miRNA transfection, cells were cultured complete medium without antibiotics. Cell culture was done at 37 °C with 5% CO2 in humidity condition.

miRNAs transfection and IFNα treatment

DLD1 dicer hypermorp cells were plated in 6-well plate for 24 h before transfection. Each miRNA was transfected into the cells at 10nM using *Trans*IT-siQuest® transfection reagent (Mirus) according to the manufacture's protocol. Twenty-four hour posttransfection, Cells were treated with 1,000U/ml Universal Type I Interferon (PBL InterferonSource) for 3 hours. After interferon treatment, cell was infected with HSV-1 at 1 M.O.I for an hour. Cell was washed once with PBS to remove unbound virus and then added 2 ml of complete McCoy's 5A medium. Cell culture medium was collected and stored at -80 until plaque assay at two days post HSV-1 infection.

HSV-1 plaque assay

Vero cells were seeded into 6-well plate to obtain 100% confluent before HSV-1 infection. The medium containing virus was diluted to several dilutions (10^{-3}-10^{-5}). Cells were incubated with HSV-1 solution for an hour (duplicate for each dilution). After infection, medium was removed and replaced with overlay medium containing 2% methylcellulose. When plaque formation (2-3 days), cell was fixed with 4% paraformaldehyde for 10 min and washed several times with PBS to remove trace methylcellulose. Cell was stained with 0.5% methylene blue.

Chapter 5: Thesis Significance and Future Work

DNA viruses, including polyomaviruses and herpesviruses, encode miRNAs, though their functions are not well defined. The primate polyomavirus SV40, a model system, encodes miRNAs. Human polyomaviruses (JCV, BKV, and MCV) are known to exist, but when I began my thesis work, it was unknown whether they also encoded miRNAs. Identifying miRNAs from additional polyomaviruses has the great potential of fostering a better understanding of polyomaviridae biology as well as viral miRNA function. Researchers have developed technologies to hunt new viral miRNAs. By bioinformatic prediction and confirmation by northern blot approach, I have identified polyomaviral miRNAs that have a functional conservation. However, this does not preclude the fact that additional miRNAs could be expressed from other genomic locations of polyomavirus. Furthermore, even though the miRNAs that have been identified from polyomaviruses have been extensively studied, it is worth exploring what miRNAs are derived from emerging polyomaviruses. For example, the WU, or KI, viruses that are possibly involved in respiratory tract disease may have a small RNA. Different approaches to miRNA hunting, such as miRNAs deep sequencing from *bona fide* polyomavirus-infected samples might yield information to answer the question and lead to more information for studying polyomaviral biology.

Another question is the function of viral miRNAs *in vivo*. In 2009, Sullivan et al., found that murine polyomaviral miRNA is not required for in vivo infection in some experimental conditions. However, it remains to be studied whether the viruses lacking

viral miRNAs have a sustainable infectivity under conditions that mimic natural transmission. In addition, it would be interesting to test whether the primate polyomavirus-virus encoded miRNAs in different genomic locations affect the viral life cycle in the *in vivo* environment. Further study may provide clues for the molecular intervention for human polyomavirus-related diseases.

RNAi serves to actively defend against viral infection in invertebrate organisms. However, researchers debate whether RNAi plays an antiviral role in mammalian cells. It has been unstudied how/if RNAi activity is modulated in response to viral infection in mammalian cells. One possible reason is that they have not been assayed under cellular conditions during an actual infection. To test if RNAi serves as an antiviral response in mammalian cells, I have developed a sensitive method to assay RNAi activity. I have demonstrated that RNAi is inhibited by the poly ADP riboylation of Argonaute protein after viral infection; antiviral response through double-stranded RNAs pathway is eventually linked to inactivation of RNAi. Completion of this study detailed a previously unknown "cross talk" between RNAi and the innate immune response in mammalian cells. It is an unresolved question how the expression of interferon-stimulated genes are promoted under the condition of global downregulation of translation after viral infection. One possible explanation suggests that miRNAs could be a possible key. miRNAs mute the target gene under normal status without triggering an antiviral response, but once the virus triggers an antiviral response, miRNA targets are relieved. Together with my Sullivan lab collegaue, I showed that the miR-17 family of miRNAs represses interferon-stimulated genes. What is necessary, however, is to determine other ISGs and miRNAs

involved in reguilation of the antiviral response. R. Kincaid has shown that miR-142 and miR-548 are also highly scored to regulate the ISGs in bioinformatics analysis (Kincaid and Sullivan, unpublished). The experimental demonstration will provide a better understanding of the function in ISG by miRNAs.

Signal transduction for RNAi repression is mediated by MAVS protein. However, effectors of this process that lie downstream of MAVs remain unknown. Interestingly, according to proteomic analysis in Li et al, 2011, the Ago2 protein is bound to TBK1 and TBK1-binding protein (SINTBAD). Since TBK1 and TBK2 are downstream effectors of MAVS, it is likely that they paly a role in the regulation that I have discovered. Also, as part of my preliminary data, a p38 MAPK chemical inhibitor blocked RNAi inactivation by pI:C. According to Zeng et al.,(2008), p38 MAPK pathways phosphorylate Ago2 protein and affect the localization of P-body or stress granule under the stress. Therefore, p38 MAPK, a downstream of MAVS signaling, may regulate RNA interference after viral infection.

Another possible experiment would be to test whether autophagy is involved in the inhibition of RNAi. Ago2 was originally known as endoplasmic or golgi–membrane-bound protein and the cellular endoplasmic membrane snatching occurs during the autophagy process after viral infection (Kim et al., 2010). According to my preliminary data, autophagy-related PI3 kinase inhibitor, 3- Methyladenine, inhibited RNAi by pI:C and sendai virus infection. It will be interesting to test whether autophagy is involved in

the inhibition of RNA using the knock-down approach by the autophagy gene siRNA in JM1 cells.

Furthermore, it would be interesting to test if RNAi is inactivated in response to microbial infection. Cellular oxidative stress inhibits miRNA activity. According to kuldow et al.,(2012) RNAi is globally repressed by microbial infection in *C elegans*. Some bacteria, infectious to the cell, cause oxidative stress. Therefore, bacterial infection may affect RNA interference. It would also be an exciting experiment to test if Toll-like receptors are involved in the inhibition of RNAi using LPS or pI:C. However, this has been a limitation in performing the pilot study because JM1 cells derived from 293 cell lack TLR 3, 4 receptors recognizing experimental ligands such as extracellular pI:C, LPS, respectively. A possible experiment can be performed after transient TLR 3 or 4 expression in JM1 cells. Or Stably TLR3 or 4 expressed 293 cell lines can be used for transient reporter assay to measure RNAi activity.

Accumulating data show miRNAs regulate immune response genes, including interferon-stimulated genes and RNAi is globally downregulated in cancer (O'Connell et al., 2012; Melo et al., 2011) . Therefore, the discovery of the small molecule modulating microRNA activity might eventually play a major role in producing a potential cancer therapy or being an intervention of viral infection. The JM1 cell lines, used for monitoring RNAi activity, could be a useful platform for performing the high throughput screening analysis to discover a small molecule modulating miRNAs activity. As a side note, 3- aminobenzamide, is clinically used for cancer therapy, in which it shows the

inhibition of RNAi depression after triggering antiviral response in cytoplasm. In summary, my study to understand a viral miRNA and RNA interference during viral life cycle may provide a clue as to we can use molecular intervention for viral infection.

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